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RESULTING FROM THE [illegible]

AND 6-(METHYLMERCAP[illegible]

IN THE TREATMENT [illegible]

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THE UNIVERSITY OF ALBERTA

THE BIOCHEMICAL BASIS FOR THE THERAPEUTIC SYNERGISM RESULTING FROM THE COMBINED USE OF 6-MERCAPTOPURINE AND 6-(METHYLMERCAPTO)PURINE RIBONUCLEOSIDE IN THE TREATMENT OF EXPERIMENTAL TUMORS

by

© MING CHANG WANG

THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "THE BIOCHEMICAL BASIS FOR THE THERAPEUTIC SYNERGISM RESULTING FROM THE COMBINED USE OF 6-MERCAPTOPURINE AND 6-(METHYLMERCAPTO)PURINE RIBONUCLEOSIDE IN THE TREATMENT OF EXPERIMENTAL TUMORS" submitted by Ming Chang Wang in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

A distinct synergism in the antitumor activities of 6-mercaptopurine (6MP) and 6-(methylmercepto)purine ribonucleoside (Me6MPR) results when these drugs are employed together in treatment of the Ehrlich ascites carcinoma (EAC). When these two agents were administered separately in low, nontoxic doses to EAC-bearing mice only minor therapeutic effects resulted. However, a potent therapeutic effect was achieved when the same dosages of 6MP and Me6MPR were administered together, as indicated by "cure" rates of 50-60% in mice so treated. The conversion of Me6MPR to Me6MP was not involved in the synergism. The agents in combination were not effective against several thiopurine-resistant tumors. Because the latter were defective in their abilities to convert 6MP or Me6MPR to nucleotides, the therapeutic result suggests that the formation of nucleotides from both agents is required for the synergistic inhibition of tumor cell proliferation.

In experiemtns with the Ehrlich ascites carcinoma _in vivo_, the formation of nucleotides from 6MP was stimulated several-fold by prior treatment with the 6MP-Me6MPR pair or by Me6MPR alone, at dosages comparable to those used in therapy. The stimulatory effect of a single dose of Me6MPR persisted up to 96 hours, evidently because Me6MPR 5'-phosphate (Me6MPRP) pools in these cells turn over at a very low rate. Thus, in combination chemotherapy

the Me6MPR component appears to enhance the synthesis of nucleotide from the 6MP present in succeeding doses of the drug mixture. Me6MPR did not stimulate 6MP anabolism in cells of the EAC-R2 subline, which are deficient in adenosine kinase as would be expected if Me6MPRP was responsible for this effect. The Me6MPR-enhanced anabolism of 6MP was not due to an increase in the concentration or activity of hypoxanthine-guanine phosphoribosyltransferase responsible for 6MP ribonucleotide synthesis and also did not stem from a reduced breakdown of 6MP-derived ribonucleotides.

Phosphoribosylpyrophosphate (PRPP) concentrations were 10-25 times higher in EAC cells following Me6MPR treatment. This finding, plus the known inhibition of glutamine-PRPP amidotransferase, indicates that Me6MPR may divert PRPP away from purine nucleotide synthesis de novo into the synthesis of 6MP ribonucleotide.

The observed stimulation of 6MP ribonucleotide formation appears to be a plausible explanation of the therapeutic synergism. However, the exact loci within the cell which are critically involved in the cytotoxic effects by the 6MP-Me6MPR pair, individually or together, are still unknown. The combined effects of both agents on glutamine-PRPP amidotransferase indicate that drug targets other than the amidotransferase must be involved in the synergism.

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List of Abbreviations

ACPR	2-amino-6-chloropurine ribonucleoside
AICAR	5-amino-4-imidazolecarboxamide ribonucleotide
AMP	adenosine 5'-monophosphate (adenylate)
ADP	adenosine 5'-diphosphate
dADP	deoxyadenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
dATP	deoxyadenosine 5'-triphosphate
CBTGR	2-amino-6-(o-chlorobenzylmercapto)purine ribonucleoside
DON	6-diazo-5-oxo-L-norleucine
FAICAR	5-formamido-4-imidazolecarboxamide ribonucleotide
GMP	guanosine 5'-monophosphate (guanylate)
GDP	guanosine 5'-diphosphate
dGDP	deoxyguanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
HPP	1-H-pyrazolo[3,4-d]pyrimidine-4-ol
IMP	inosine 5'-monophosphate (inosinate)
ip	intraperitoneal
Me6MP	6-(methylmercapto)purine
Me6MPR	6-(methylmercapto)purine ribonucleoside (6-methylthioinosine)
Me6MPRP	6-(methylmercapto)purine ribonucleoside 5'-monophosphate (6-methylthioinosinate)
6MP	6-mercaptopurine

6MPR	6-mercaptopurine ribonucleoside (thio-inosine)
6MPdR	6-mercaptopurine deoxyribonucleoside
6MPRP	6-mercaptopurine ribonucleoside 5'-monophosphate (6-thioinosinate)
NAD	nicotinamide adenine dinucleotide
PCA	perchloric acid
Poly A	polyadenylate
PRA	5-phosphoribisyl-1-amine
PRPP	5-phosphoribosyl-1-pyrophosphate
SAMP	succinyladenylate (adenylosuccinate)
6TG	6-thioguanine
THF	tetrahydrofolate
6TXMP	6-thioxanthylate

I. INTRODUCTION

A. Combination Chemotherapy

In the development of an effective chemotherapy of cancer, an important line of effort is represented by attempts to increase the activity of known drugs through improvements in the method of application. While variations in dosage levels and treatment schedules are rather obvious means to this end, combination chemotherapy appears to have a much larger potential value as a means of extending the usefulness of our present antitumor drugs, because particular durg combinations show "greater than additive" effects. Drug combinations have been used with very encouraging results in the treatment of human leukemias. Treatment of acute leukemia in children with the "VAMP" combination (vincristine, amethopterin, 6-mercaptopurine, and prednisone) has induced a high proportion of complete remissions of very substantial duration (in one study the median time to relapse was 150 days (1)); the same combination treatment has also been used in the treatment of adult acute myelogenous leukemia, with a resulting remission rate of 70% (2).

A number of studies with experimental tumors have shown clearly that distinct therapeutic advantage results from the use of certain drugs in combination; these experiments with laboratory animals have provided a quantitative demonstration of potentiation of therapeutic effects, which

is not really possible in clinical testing. That the tumor inhibition achieved by one drug may be potentiated by the presence of a second drug is apparent in the following examples. A treatment schedule with 6-chloropurine, which was without effect against either the Ehrlich ascites carcinoma (EAC) or Sarcoma 180, markedly enhanced the antitumor activity of azaserine when the two drugs were used together against these tumors (3). Similarly, 1-H-pyrazolo[3,4-_d_]-pyrimidine-4-ol(HPP) was inactive against Adenocarcinoma 755, yet when used in combination with either 6-(methylmercapto)-purine (Me6MP), 6-(propylmercapto)purine, or 6-chloropurine, HPP increased the antitumor effect of the latter (4). After treatment of EAC-bearing mice with combinations of azaserine and 6-thioguanine (6TG), with each at dosages that produced only minor therapeutic effects when employed separately, a high proportion of the animals became long-term survivors, that is, they were apparently "cured" (5).

Such potentiation[1] represents a highly valuable amplification of drug effect. Only particular durg combinations show such "greater than additive" effects.

The therapeutically potentiating drug pair, 6-mercaptopurine and 6-(methylmercapto)purine ribonucleoside (Me6MPR)

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[1] The term "potentiation" is used in this thesis interchangeably with "synergism" with the meaning that the inhibition of tumor cell proliferation which results from the concurrent administration of two drugs, is greater than the sum of the effects of each drug given separately.

was the principal concern of this study. Our initial finding that the combination of these agents resulted in a distinct potentiation of their inhibitory effects toward the Ehrlich ascites carcinoma *in vivo* has been reported (6). This thesis will describe experiments with these and related drugs which demonstrate the potentiation and which also bear on its biochemical mechanism. A major objective of this study was to explain in biochemical terms this enhancement of therapeutic effect; experiments on the metabolism of these drugs in Ehrlich ascites tumor cells showed that Me6MPR enhanced the synthesis of 6MP ribonucleotide, the "active form" of 6MP.

Therapeutic potentiation with this drug combination was also demonstrated independently by Schabel *et al.* (7) in experiments with mouse leukemia L1210 *in vivo*. Paterson and Moriwaki (8) have shown recently that the 6MP-Me6MPR pair potentiate in inhibiting the proliferation of lymphoma L5178Y cells either *in vivo* or in culture. Use of this drug pair in the treatment of human acute leukemia by Bodey *et al.* (9) has yielded promising results; however, these trials were not intended to demonstrate therapeutic potentiation.

B. Biochemical Properties of 6-Mercaptopurine and 6-(Methylmercapto)purine Ribonucleoside

The metabolism, biochemical effects, and possible mechanisms of action of 6MP and Me6MPR are reviewed below in order to permit consideration of the biochemical mechanism of 6MP-Me6MPR synergism.

1. <u>6-Mercaptopurine(6MP)</u>

The synthesis of this compound was reported by Elion *et al.* (10) in 1952 and the following year Clarke *et al.* (11) described the antitumor activity of 6MP against Sarcoma 180. Since then, 6MP has been tested against many neoplasms; in a survey of the literature to 1963, Hirschberg (12) stated that 6MP was active against 75 of 120 tumors tested.

6MP has a very important place in the chemotherapy of human neoplastic disease, both in the historical sense (13) and in current clinical practice (14). 6MP is currently used in the treatment of acute lymphocytic leukemia, chronic leukemia (14), and as one component of the "VAMP" combination (1) in the treatment of acute leukemia in children (1) and adults (2).

The ability to suppress the immune response is another highly valuable property of 6MP; today, 6MP and its derivative, Imuran[2], are widely used to suppress homograft rejection (14).

Because of clinical usefulness, the biochemical properties of 6MP have been extensively studied.

a) <u>Catabolism</u>

As shown in Figure 1, there are two established catabolic pathways for 6MP. In the first pathway, 6MP is oxidized by xanthine oxidase to 6-mercaptopurine-8-ol (6MP-8-OH) and 6-mercaptopurine-2,8-diol (6MP-2,8-diOH); this occurs in microbes (15), animals (16-18), and man (19). In

---

[2] 6-[(1-Methyl-4-nitro-5-imidazolyl)thio]purine

FIGURE 1

Catabolism of 6-mercaptopurine

Hypoxanthine ⟶ Xanthine ⟶ Uric acid

↑ * (from 6MP)

6MP** ⟶ 6MP-8-OH ⟶ 6MP-2,8-diOH ⟶ Sulfate + other products

6MP ⟶ Me6MP

6MP-2,8-diOH ⟶ Me6MP-2,8-diOH

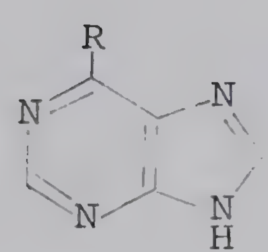
Sulfate + other products ⟵ Me6MP ⟶ Me6MP-8-OH ⟶ Me6MP-2,8-diOH

Me6MP-8-OH ⟶ 6-(Methylsulfinyl)-purine-8-OH

Me6MP-8-OH ⟶ Me6MP-8-OH glucuronide

* Not conclusively demonstrated.

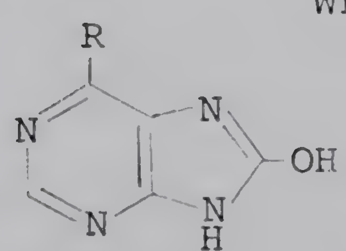
** The structural formulae of 6MP and its metabolites are as follows:



When R = OH, hypoxanthine

SH, 6-mercaptopurine (6MP)

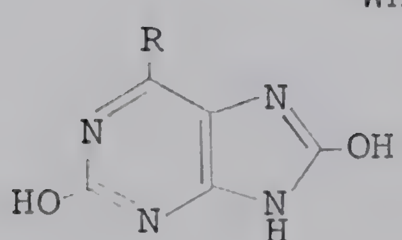
SCH_3, 6-(methylmercapto)purine (Me6MP)



When R = SH, 6-mercaptopurine-8-ol (6MP-8-OH)

SCH_3, 6-(methylmercapto)purine-8-ol (Me6MP-8-OH)

$SOCH_3$, 6-(methylsulfinyl)purine-8-ol



When R = OH, uric acid

SH, 6-mercaptopurine-2,8-diol (6MP-2,8-diOH)

SCH_3, 6-(methylmercapto)purine-2,8-diol (Me6MP-2,8-diOH)

mice and rats, 6MP-2,8-diOH may be further catabolized with the release of sulfate (17,20), or methylated to form 6-(methylmercapto)purine-2,8-diol (Me6MP-2,8-diOH) (18,20). Uricase is involved in the degradation of 6MP-2,8-diOH to sulfate (21); this process evidently does not occur in man, who lacks this enzyme (22). Further to this point, the xanthine oxidase inhibitor, HPP, blocks the formation of 6MP-2,8-diOH in man without affecting sulfate formation from 6MP (23); thus, the degradation of 6MP to sulfate in man occurs by a route other than the xanthine oxidase-uricase pathway (see below).

In the other catabolic pathway, 6MP is methylated to form 6-(methylmercapto)purine (Me6MP) which can then be degraded to yield sulfate, or oxidized by xanthine oxidase to form 6-(methylmercapto)purine-8-ol (Me6MP-8-OH) and Me6MP-2,8-diOH. Catabolism by this pathway takes place in the mouse, the rat, and apparently in man (17-20,23,24). 6-(Methylsulfinyl)purine-8-ol and Me6MP-8-OH glucuronide have been found in human urine as metabolites of 6MP (23).

It has been demonstrated that the purine ring of 6MP may be incorporated into polynucleotide adenine and guanine in *Streptococcus faecalis* (25), *Bacillus cereus* (15) and Adenocarcinoma 755 (26). Evidently, the 6MP molecule is dethiolated at some stage to form hypoxanthine, but this does not necessarily occur at the free base level. In this context, it may be noted that 6MP was also incorporated into nucleic acid adenine and guanine in *Escherichia coli*, but dethiolation occurred only after 6MP had been conver-

ted to 6MPR 5'-monophosphate (6-thioinosinate, 6MPRP) (27).

b) Anabolism

Figure 2 summarizes the anabolism of 6-mercaptopurine.

Purine nucleoside phosphorylase is a widely distributed enzyme (28) which forms both 6MP ribonucleoside (6-thioinosine, 6MPR) and 6MP deoxyribonucleoside (29-32); the latter is also a product of the bacterial deoxyribosyltransferase reaction (33).

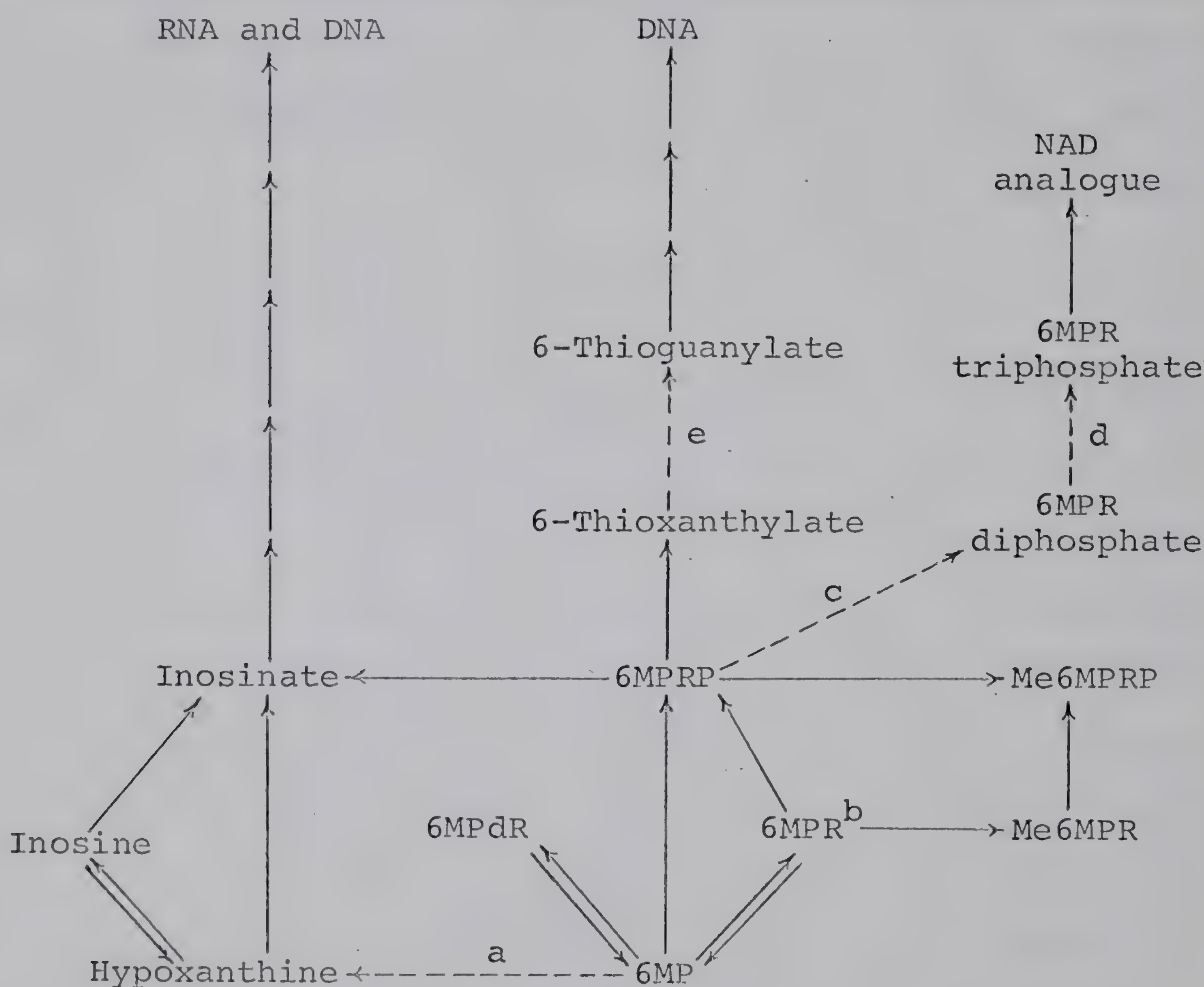
A kinase activity by which the 5'-monophosphate of 6MPR is formed has been detected in Ehrlich ascites tumor cells[3] and in a thioguanine-resistant subline, ETGR II (34); inosine kinase is apparently the enzyme responsible. The sulfhydryl group of 6MPR may be methylated by a methyltransferase to form 6-(methylmercapto)purine ribonucleoside (Me6MPR)(35,36,52);the latter is a substrate for adenosine kinase (37,38,39). Formation of the 5'-monophosphate derivative of Me6MPR has been shown in EAC *in vivo* (38), H.Ep. No. 2 in culture (37,40) and in yeast cells (37).

6MPRP can also be formed directly from 6MP and PRPP (41-45). It has been shown that partly-purified preparations of the hypoxanthine-guanine phosphoribosyltransferase which are devoid of adenine phosphoribosyltransferase acitivty, accept 6MP as a substrate to form 6MPRP (41,43). Further, thiopurine-resistant cells which lack hypoxanthine-guanine phosphoribosyltransferase have been shown incapable of converting 6MP to 6MPRP (44,46,47). For these reasons, the hypoxanthine-guanine enzyme is believed to be respon-

[3] I. C. Caldwell, unpublished results.

FIGURE 2

Anabolism of 6-mercaptopurine



[a] Not conclusively demonstrated.

[b] Abbreviations: R, ribosyl; dR, deoxyribosyl; RP, ribosyl 5'-monophosphate.

[c] Demonstrated with a spectrophotometric method (53), but the diphosphate product was not actually isolated.

[d] Not yet demonstrated.

[e] Inferred because 6MP was incorporated into DNA as 6-thioguanine.

sible for the PRPP-dependent formation of 6MPRP.

It has been shown that the inosinate dehydrogenase of Aerobacter aerogenes will accept 6-thioinosinate as a substrate to form 6-thioxanthylate (6TXMP) (48); Atkinson et al. (49) have demonstrated that in Ehrlich ascites tumor cells 6TXMP is a late metabolite of 6MP, and is apparently formed from 6-thioinosinate. Me6MPR 5'-monophosphate (Me6MPRP) has been found as a matabolite of 6MP in cells of the Ehrlich ascites carcinoma (50), Adenocarcinoma 755, mouse leukemia L1210, and H.Ep. No. 2 (35,51); temporal relationships suggest that 6-thioinosinate is the immediate precursor of Me6MPRP. Remy (36,52) has shown that both 6-thioinosine and 6-thioinosinate are substrates for a methyltransferase activity in animal tissues. In addition, 6-thioinosinate is converted to a number of uncharacterized nucleotides in Ehrlich ascites tumor cells[3]. In E. coli, 6-thioinosinate was dethiolated to inosinate (27).

In Adenocarcinoma 755, 6MP was incorporated into DNA as thioguanine (26), indicating that thioguanine ribo- and deoxyribonucleotides were intermediate metabolites in this process; however, these analogue nucleotides have not yet been demonstrated in 6MP-treated cells.

6MPRP is apparently a substrate for purine nucleoside monophosphate kinase; Way et al. (53) have concluded that phosphorylation of 6MPRP was catalysed by a partly purified kinase from pork kidney. However, it may be noted that this conclusion was based on ATP consumption data; 6MP-containing products were not isolated in this experiments.

Di- and triphosphates of 6MPR have not been found in the acid-soluble fraction of 6MP-treated EAC (54), nor in ethanol extracts of 6MP-treated L1210 cells (44). Indeed, to date the isolation of the enzymatically synthesized 6MPR di- or triphosphate has not been reported. Atkinson et al. (55) have shown that chemically synthesized 6MPR triphosphate is incorporated into an NAD analogue.

The possibility of 6MP incorporation into polynucleotide is important in the consideration of action mechanisms; however, this has been difficult to assess because 6MP metabolites are known to be incorporated and 6MP can bind non-enzymatically to polynucleotides. It has been reported that radioactivity from 6MP-^{35}S or 6MP-8-^{14}C was incorporated into the nucleic acid fraction of mouse tissues, both normal and neoplastic (16,56). However, nucleates isolated from *B. cereus* or P388 murine lymphocytic leukemia cells that had been incubated with ^{14}C- or ^{35}S-labelled 6MP, contained the isotopic label, but did not contain 6MP *per se* (15,57). The ^{14}C-label in *B. cereus* nucleates was associated with adenine and guanine while ^{35}S in nucleate preparations from the leukemia cells possibly came from contaminating ^{35}S-labelled protein. At this point, it is worth mentioning that incorporation of intact 6MP molecules into the polynucleotide structure would require the biosynthesis of thioinosine polyphosphates, according to our present understanding of polynucleotide synthesis. However, this has not yet been conclusively demonstrated, as noted above. It has been clearly shown that 6MP can bind non-

enzymatically to yeast RNA, evidently by a metal bridge bond (58); at least part of the isotopic label from 6MP-^{35}S associated with RNA isolated from mouse and rat livers may be attributable to nonenzymatic binding involving metal complexes (59). Because of this nonenzymatic binding, a valid demonstration of 6MP incorporation requires evidence that the analogue was actually incorporated into the polynucleotide chain. Such evidence was obtained by Scanell and Hitchings (26) in experiments in which mice bearing 6MP-resistant Adenocarcinoma 755 were injected with 6MP-8-^{14}C; when DNA of the tumor was isolated and treated successively with pancreatic deoxyribonuclease and snake venom, radioactivity was found to be mainly associated with thioguanine deoxyribonucleoside indicating that a 6MP metabolite had been incorporated into DNA in nucleotide linkage.

c) Metabolic effects

The principal metabolites of 6MP are thioinosinate and its methyl derivative, and thioxanthylate. However, as noted above, a number of other derivatives are also formed; hence, it should not be unexpected that 6MP produces a complex of biochemical effects.

Effects on nucleotide and nucleic synthesis: In early attempts to identify the metabolic effects of 6MP, it was recognized that 6MP inhibited the incorporation of *de novo* purine precursors, such as glycine, into nucleic acid purines of mouse tumors (60,61); the incorporation of preformed bases was also inhibited (60). These effects, which were detected at the nucleic acid level, are undoubtedly a

reflection of primary effects now known to occur at the level of nucleotide metabolism. A number of enzymes involved in the synthesis and interconversion of purine ribonucleotides have been shown to be inhibited by 6MP or its metabolites; Figure 3 summarizes these facts.

It may be noted in Figure 3 that nucleotide metabolites of 6MP inhibit enzymatic steps leading to AMP and GMP. The first step of IMP synthesis _de novo_ is inhibited by Me6MPRP and 6MPRP; the latter inhibits both steps in the conversion of IMP to AMP and, as well, the formation of XMP. These inhibitions have been detected and studied in enzymatic experiments; however, their relevance to the growth inhibitory effects of 6MP in living cells is a matter of great uncertainty (see section d).

Two points about Figure 3 should also be noted: (i) although the polymerization of ADP (reaction k) and the synthesis of NAD (reaction m) were inhibited _in vitro_ by 6MPR di- and triphosphate, respectively, the _in vivo_ formation of the latter nucleotides is questionable, (ii) even though the glutamine-PRPP amidotransferases (reaction a) from pigeon liver (62) and Adenocarcinoma 755[4] were inhibited by 6MPRP, the partly purified amidotransferase from EAC cells was not inhibited by 6MPRP[5].

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[4] L. L. Bennett, Jr., personal communication.

[5] M. R. Atkinson, personal communication.

FIGURE 3

Inhibition in nucleotide metabolism exerted by 6-mercapto-purine and its metabolites

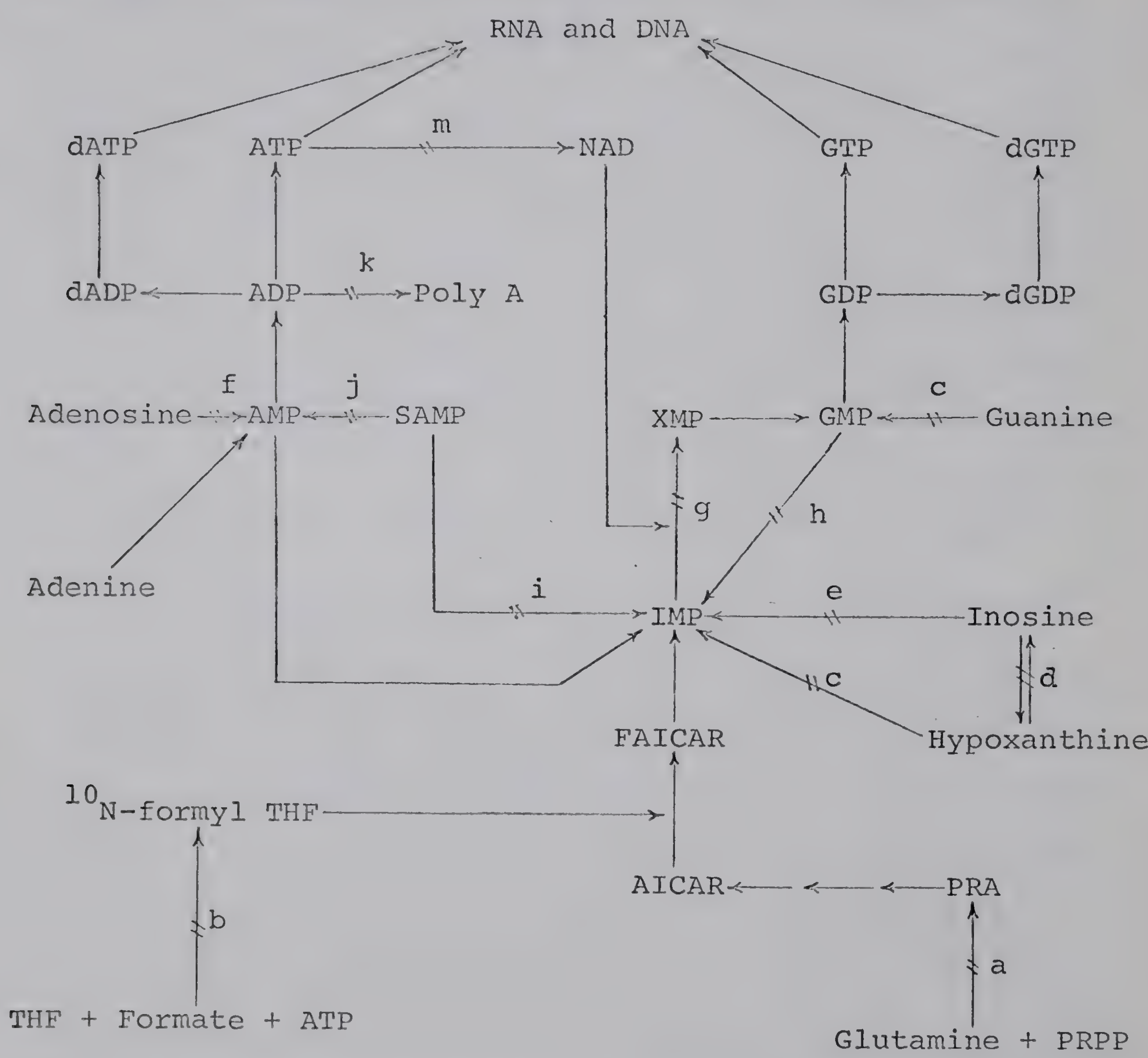

FIG. 3 (Continued)

Reaction	Enzyme	Inhibitor
a	Glutamine-PRPP amidotransferase	6MPRP, Me6MPRP[4,5] (62)
b	Formate-activating enzyme	6MP, 6MPRP (64)
c	Hypoxanthine-guanine phosphoribosyltransferase	6MP, 6MPRP (43, 65,66)
d	Purine nucleoside phosphorylase	6MP, 6MPR (67,68)
e	Inosine kinase	6MPR (69)
f	Adenosine kinase	Me6MPR (37)
g	IMP dehyrogenase	6MPRP (48,71,72)
h	GMP reductase	6MPRP (73)
i	Adenylosuccinate synthetase	6MPRP (74,75,76)
k	Polynucleotide phosphorylase	6MPR diphosphate (78)
m	Nicotinamide adenylyltransferase	6MPR triphosphate (55,107)

<u>Other effects</u>: Antibody formation (14), bacterial flagella formation (80), and the induction of a number of enzymes (81-83) are blocked by 6MP; Elion (84) has discussed possible mechanisms by which 6MP may block the synthesis of protein and has suggested messenger RNA or ribosomal RNA as a possible target of 6MP.

Lipogenesis in embryo skin fibroblasts is inhibited by 6MP (85), as are a number of acetate-utilizing reactions in other systems (86-88). It has been suggested that interference with the synthesis or function of coenzyme A might be responsible for such 6MP effects (85-89); however, in *B. cereus* neither the content of coenzyme A, nor the utilization of acetate was affected by 6MP (90).

There are many reports concerning the effect of 6MP on carbohydrate metabolism (91-95), but the mechanism of this effect has not been investigated in depth. Presumably, 6MP could produce such an effect by inhibiting the synthesis of purine-containing coenzymes involved in carbohydrate metabolism.

In addition, it has been reported that 6MP inhibits xanthine oxidase (96), catalase (97), the N-methylation of nicotinamide (98), and the incorporation of diaminopimelic acid into bacterial cell walls (80).

d) <u>Mechanism of action</u>

The mechanism of the growth inhibitory effects of 6MP cannot yet be attributed to interference at any particular enzymatic loci. However, it is generally accepted that the formation of thioinosinate in an essential step in the

the manifestation of the inhibition. This idea derived from a number of instances in which cell lines that were selected for resistance to 6MP were found to have impaired ability to form the analogue nucleotide (99,100). Although thioinosinate has been called the "active form" of 6MP, it should be recognized that other metabolites of thioinosinate may also be responsible for, or contribute to, growth inhibitory effects.

Attempts have been made to evaluate the contribution of particular, 6MP-derived enzymatic inhibitions to the growth inhibitory effects of 6MP and several workers have suggested "loci of action" for 6MP; these are discussed below.

<u>Inhibition of nucleotide metabolism</u>: On the basis of estimates of intracellular concentrations of enzymatic substrates and of 6MP or its ribonucleotide under chemotherapeutic conditions, Elion (84) has concluded that the reactions catalysed by adenylosuccinate synthetase, inosinate dehydrogenase, and glutamine-PRPP amidotransferase were the most vulnerable of those known to be impaired by 6MP. However, in assessing the critical metabolic effects of 6MP in individual cell systems, several investigators have perceived different "loci of action"; these may well be characteristics of the particular cells studied.

Hakala and Nichol (101) found in their initial studies that inhibition by 6MP of cultured HeLa and Sarcoma 180 cells could be reversed by adenine, hypoxanthine, and their nucleosides and nucleotides; however, reversal by adenine compounds was independent of the molar ratio of inhibitor

to reversing agent, whereas reversal by hypoxanthine compounds was dependent upon this relationship. These observations were interpreted to mean that interference with the formation of adenosine phosphates from IMP was responsible for the growth inhibitory action of 6MP.

A different site of action was indicated in work by Carey and Mandel (90), who observed that adenine and hypoxanthine competitively reversed the inhibition of _B. cereus_ growth caused by 6MP, while guanine reversed this in a noncompetitive manner; this result suggested that inhibition of the conversion of inosinate to guanylate is more important for the growth inhibitory effect of 6MP than is the conversion of inosinate to adenylate.

It is evident in the foregoing examples that known loci of sensitivity to 6MP differ in importance from one cell type to another; this consideration also applies to the following discussion of the significance of the inhibition of glutamine-PRPP amidotransferase in the action mechanism of 6MP.

Bennett _et al_. (102) found that in EAC, L1210 and Sarcoma 180 cells, glutamine-PRPP amidotransferase was much more sensitive to 6MP than the enzymes involved in purine nucleotide interconversions. Subsequently, Hakala and Nichol (103) found in their further studies on the inhibition of growth of cultured Sarcoma 180 and HeLa cells by 6MP that blockade of glutamine-PRPP amidotransferase was of primary importance for the growth inhibitory effects of 6MP. However, LePage and Jones (5) found a lack of correla-

tion between inhibition of the amidotransferase and the growth inhibition caused by thiopurines: the Mecca lymphoma is intrinsically resistant to 6MP, yet the glutamine-PRPP amidotransferase activity of Mecca cells was inhibited by 6MP to a greater extent than were the amidotransferase activities of EAC and Adenocarcinoma 755, both of which are very sensitive to 6MP.

Interference with the function of nucleic acid: Interference with the synthesis and function of nucleic acid, as a consequence of incorporation of 6MP, has been postulated as a mechanism of action of 6MP (19). Although incorporation of the 6MP molecule *per se* into polynucleotides has not yet been conclusively demonstrated, it has been shown that 6MP is incorporated into DNA as 6-thioguanine. As noted previously, the synthesis of some specific proteins, notably the induction of certain enzymes, is blocked by 6MP; such an effect suggests either the function or synthesis of RNA was interfered by the drug, because it is well known that various species of RNA are involved in the complex process of protein synthesis. Incorporation of 6MP or its metabolites is perhaps the most obvious way to impair RNA function or synthesis, however, attachment of the thiopurine to nucleates might have such an effect.

Preceding sections have discussed the effects of 6MP on the intermediary metabolism of nucleotides; intuitively, one might expect these to be manifested as "acute" effects on the proliferation of cells. A different type of effect has been noticed in experiments with cultured cells;

reductions in proliferation rates appear several generations after exposure to 6MP and are reminiscent of the effects of bromodeoxyuridine incorporation into DNA (104). Such delays might reflect the time required for expression of damage or faults introduced into DNA in the presence of 6MP.

Tomizawa and Aronow (105) observed that upon brief exposure to low concentration of 6MP (about 10^{-6} M), mouse fibroblasts in culture multiplied for several days and then a decline in cell numbers took place. It was postulated that the delayed toxic effect might be caused by the incorporation of 6MP into DNA. Delayed toxic effects have also been seen with Sarcoma 180 cells _in vivo_ (11), and with L5178Y lymphoma (8) and HeLa cells (106) in culture. It is of interest to note that the growth curve of HeLa cells which had been exposed to 6MP briefly was similar to that of irradiated HeLa cells (106).

In the experiments of Tomizawa and Aronow (105), the growth of mouse fibroblasts was inhibited without delay at high 6MP concentrations (about 10^{-3} M), suggesting that acute drug effects in addition to the presumed effects at the polynucleotide level may be involved. It is likely that growth inhibition by 6MP involves interference with nucleic acid function together with various metabolic effects; effects at both levels could very well be promoted by enhancement of 6MP anabolism. The latter idea will be developed subsequently in this work.

Interference with synthesis and function of coenzymes: The suggestion of Atkinson _et al_. (107) that 6MP might

interfere with growth of cells through inhibition of NAD synthesis, was based on the facts that (i) 6-thioinosine triphosphate inhibited nicotinamide adenylyltransferase isolated from pig liver nuclei, (ii) in a number of tumors the activity of the adenyltransferase, as well as the NAD content per nucleus, is considerably less than that in normal tissues, and (iii) symptoms similar to those of nicotinamide deficiency develop sometimes in humans who have received prolonged treatment with 6MP. However, Elion (84) has pointed out that inhibition of this enzyme is not likely to occur because the cellular concentration of ATP would be much higher than 6-thioinosine triphosphate, even if the latter compound did form in 6MP-treated cells. In addition, Carey and Mandel (90) have shown that 6MP hindered the growth of _B. cereus_ without affecting NAD synthesis.

Biesele (108) found that 6MP decreased the incidence of mitosis and increased the number of degenerating nuclei in cultured Sarcoma 180 and mouse fibroblasts. Of the purine derivatives tested to reverse this effect, the most effective was coenzyme A. However, in _B. cereus_ neither the synthesis of coenzyme A, nor its function, as judged by acetate utilization, were affected by 6MP concentrations that inhibited growth (90).

Thus, loci of action for 6MP which are important in one cell type are less important in other cell types, and a mechanism for the cytotoxic action of 6MP in any cell type still remains to be discovered. Perhaps this should be no wonder because 6MP inhibits many enzymes, and the survival

value, as well as the sensitivity to 6MP, of each of these enzymes likely varies from one cell type to another, being influenced by factors such as intracellular concentrations of substrate, inhibitor, and enzyme.

2. 6-(Methylmercapto)purine ribonucleoside (Me6MPR)

The antitumor activity of this derivative of 6MP has been demonstrated against Sarcoma 180, Adenocarcinoma 755, mouse leukemia L1210 (109), and Ehrlich ascites carcinoma (6,99). This compound was active against a 6MP-resistant line of mouse leukemia L1210 (40); however, in clinical trials against 6MP-resistant acute myelocytic and lymphocytic leukemias this drug was not effective (110).

a) Metabolism

Me6MPR is converted to the 5'-phosphate by neoplastic cells and by mouse and human tissues, apparently by adenosine kinase (37,39,40). The 5'-phosphate is the principal metabolite of Me6MPR in Ehrlich ascites tumor cells (38); it is noteworthy that the di- and triphosphate derivatives have not been found in cells which contain substantial concentrations of Me6MPR 5'-phosphate (38,40). The data of Paterson (29), Caldwell et al. (38), and Bennett *et al*. (40) indicate that Me6MPR is not subject to phosphorolytic cleavage, although Krenitsky *et al*. (68) have shown that this does take place at a low rate in human erythrocytes.

b) Metabolic effects

Me6MPR was reported to inhibit nucleoside metabolism in intact cells (111); in this respect, Me6MPR is acting as the simplest member of a family of compounds (6-(alkyl-

mercapto)purine ribonucleosides) which inhibit the transport of nucleosides across cell membranes (112). Nucleoside transport is a "carrier"-mediated process (113-115), the most potent known member of the inhibitor family, p-nitrobenzylthioguanosine, appears to interact irreversibly with the membrane "carrier"[6]. Me6MPR has little or no effect on the purine nucleoside phosphorylase reaction (68, 111,112). The ability of Me6MPR to interfere with nucleoside transport is not related to its antitumor activity, judging from the fact that this effect is mainifested in Me6MPR-resistant and sensitive cells alike.[7]

Early studies by Henderson (116) showed that Me6MPR blocked the azaserine-induced accumulation of FGAR in Ehrlich ascites tumor cells *in vitro*, suggesting that an enzymatic step prior to the formation of FGAR in purine synthesis *de novo* was inhibited. This observation has been confirmed with the cells of Ehrlich ascites carcinoma (63, 79), leukemia L1210, and H.Ep.No.2 (40). The *in vivo* incorporation of glycine, a purine precursor, into acid-soluble adenine compounds of various mouse tissues is also inhibited by Me6MPR (117). Henderson and Khoo (79) showed that glutamine-PRPP amidotransferase in Ehrlich ascites tumor cells *in vitro* was inhibited by Me6MPR; the real inhibitor is apparently Me6MPR phosphate because in cells unable to phosphorylate Me6MPR, purine synthesis *de novo* is not inhibited by

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[6] J.M. Oliver, Master of Science thesis, The University of Alberta (1968).

[7] A.R.P. Paterson and A.I. Simpson, unpublished results.

the drug (63). It has been demonstrated that Me6MPR phosphate is a potent inhibitor of partly purified glutamine-PRPP amidotransferases from Adenocarcinoma 755[4] and the Ehrlich ascites carcinoma[5]. Thus, Me6MPR phosphate, like certain other analogue nucleotides, acts as a "pseudofeedback inhibitor" (62) on the amidotransferase, the first enzyme in the purine *de novo* synthetic pathway.

c) Mechanism of action

Caldwell *et al*. (63) showed that cells of a Me6MPR-resistant subline of the Ehrlich ascites carcinoma were deficient in the kinase that phosphorylated Me6MPR. A similar observation has been made by Bennett *et al*. (37) with several Me6MPR-resistant lines of cultured H.Ep.No.2 cells. These data suggest that the phosphorylation of Me6MPR is an essential step in the manifestation of the growth inhibitory effect of this analogue ribonucleoside. Since glutamine-PRPP amidotransferase in cells is strongly inhibited by Me6MPR, as has been mentioned, Me6MPR might inhibit growth of tumor cells by inhibiting purine synthesis *de novo*, although proof of this statement does not presently exist.

C. Objectives in this Study

This study was aimed at explaining in biochemical terms how 6MP and Me6MPR synergize when used together in inhibiting growth of the Ehrlich ascites carcinoma.

The drug transformations in the tumor cells that were needed for the manifestation of synergism were studied by

testing the drug combination against several thiopurine-resistant tumors which had defects in converting one drug or the other into nucleotide. It was found that formation of the nucleotide derivative of both drugs was essential to the synergism. Because of this finding the effect of one drug upon the conversion of the other to its nucleotide form was investigated. The result showed that nucleotide synthesis from 6MP was enhanced by Me6MPR, whereas the phosphorylation of Me6MPR was not affected by 6MP. The biochemical basis for the enhancement of 6MP ribonucleotide synthesis appeared to be due to an increased availability of PRPP.

Finally, correlation between enhancement of nucleotide synthesis from 6MP and the synergism in the antitumor effect was demonstrated.

II. MATERIALS AND METHODS

A. Tumors and Their Maintenance

The tumors used in these studies are listed in Table I, along with their sources and some of their characteristics. The Ehrlich ascites tumor and its sublines were maintained by weekly transplantation (118) of 5 to 6 million tumor cells into Ha/ICR mice obtained from our own colony, or from the A.R. Schmidt Co., Madison, Wisc. Lymphoma L5178Y was maintained by weekly transplantation of 10 million cells into the peritoneal cavity of male BDF_1 mice obtained from Microbiological Associates, Inc., Bethesda, Md.

B. Chemotherapy Experiments

1. Implantation of tumor

Mice were implanted intraperitoneally with 5 to 6 million ascites tumor cells each(taken from a pool of cells collected after 7 days of growth in donor mice) and were randomly assigned to treatment groups (10 mice) which were then weighed in total. Drug doses were based on the average weight of the mice in each treatment group at the start of experiment; unless otherwise specified, variations among individual mouse weights were within a 5-gram range.

2. Administration of drug

Unless otherwise specified, antitumor agents were administered by intraperitoneal (ip) injection at 24 hour inter-

TABLE I

Tumor lines: origins and characteristics

Tumor	Source	Drug resistance: Selecting agent	Drug resistance: Colaterally resistant to	Drug nucleotide formed: 6MP	Drug nucleotide formed: Me6MPR
EAC	Stock*			Yes	Yes
EAC-R1	Paterson (118)	6MP	Me6MPR	Trace	Yes
EAC-R2	Caldwell *et al.* (63)	Me6MPR	6MP	Yes	Trace
ETGRI	LePage (119)	6TG	6MP, Me6MPR	Yes**	Yes
ETGRII	LePage (119)	6TG	6MP, Me6MPR	No	Yes
L5178Y	Sartorelli†			Yes	Yes

* This hypotetraploid (73 to 74 chromosomes) line of the Ehrlich ascites carcinoma was obtained by Dr. Paterson in 1956 from Dr. C.C. Stock, Sloan-Kettering Institute for Cancer Research, New York.

** Although nucleotide is formed from 6MP in these resistant cells, the amount is only 30% of that formed in EAC cells.

† Obtained from Dr. A.C. Sartorelli, Yale University School of Medicine.

vals, starting 24 hours after tumor implantation, to a total of 5 doses. 6MP was dissolved in 0.154 M saline in those experiments of Section III E which used packed cell weight to evaluate treatment effects; in other experiments, 6MP was administered as a fine suspension in saline. Other drugs were dissolved in saline.

3. Evaluation of chemotherapy

Two methods were employed to assess the effects of drug treatment on the proliferation of tumor cells in implanted mice: (a) the mass of tumor cells recoverable from the peritoneal cavity was determined at a standard time after completion of a course of therapy, or (b) the mean survival times of treated and control mice were compared.

Mass of tumor cells: Mice were killed 7 days after implantation of tumor, that is, 2 days after completion of therapy. To facilitate the recovery of tumor cells from the peritoneal cavity, immediately after death each mouse received by ip injection 2 ml of heparin solution (10 mg heparin in 100 ml of 0.154 M saline). The ascitic fluid of each mouse was drained through an abdominal incision into a tared 12 ml conical centrifuge tube; saline rinsings of the peritoneal cavity were added and the tubes were centrifuged at 2,400 x g for 10 minutes. After the supernatant fluids were discarded, tube walls above the pellets were wiped dry,and the weight the cell sediments were determined.

In one experiment, packed cell volumes were determined. The ascitic fluids were collected in Kolmer tubes, using the procedure described above. The tubes were then centrifuged

at 2,300 x g for 3 minutes and the volumes of packed cells were read from the scale of the tube.

Survival time: After chemotherapy treatments were completed, cages were inspected daily at a set time. The numbers of mice surviving and dead were recorded; as well, mice were weighed 1, 4, and 7 days after treatment was finished.

In occasional experiments, control mice (implanted with tumor, but treated with saline only) survived and apparently tumor-free at the end of the experiment. The aggregate experience in this work was that about 2% of tumor implants in control mice did not "take"; in any single experiment the highest percentage of such "no takes" was 10%. Thus, the appearance of a single long-term survivor in a treatment group of 10 mice was not considered significant.

C. Biochemical Experiments

1. Injection of drugs and extraction of tumor cells

Drugs were dissolved in 0.154 M saline and injected into the ascitic fluid of tumor-bearing mice; unless otherwise specified, mice were used on the 7th day after tumor implantation. Immediately after injection, collodion was applied to the site of injection to prevent leakage of ascitic fluid. At specified times after drug injection, mice were killed, and ascitic fluids from 1 to 3 mice were drained into a 40 ml centrifuge tube containing 2 ml heparin solution (10 mg heparin in 100 ml of 0.154 M saline). After centrifuging

at 2,400 x g for 10 minutes at 4°, the supernatant was discarded and the wet weight of packed cells was determined.

Cell sediments were mixed with 2 volumes of cold 0.4 M perchloric acid (PCA) and kept for 15 minutes in ice water with occasional mixing; the PCA extract was recovered by centrifugation and the residue was extracted again with 1 volume of 0.2 M PCA. Both extracts were pooled and neutralized with 20% potassium hydroxide. The precipitate formed was removed by centrifugation and the neutralized extracts were stored at -20° until analysed.

2. Analytical procedures

Determination of "total nucleotides": The term "total nucleotides", as used herein, refers to the unresolved group of nucleotide metabolites derived from 6MP, or from other purine bases. Paper chromatography in solvent A or B (see page 30) provided group separations of bases, nucleosides, and nucleotides and was the basis for the determination of "total nucleotides". The entire nucleotide area of the chromatogram was assayed for radioactivity.

For analyzing "total nucleotides" formed from ^{14}C-labelled adenine, guanine and 6MP, measured volumes (50 to 100 µl) of neutralized PCA extracts were chromatographed using solvent A, together with non-radioactive "carriers" representing the appropriate base, nucleoside and nucleoside monophosphate. An identical volume of each sample was applied in the same chromatogram lane at a point beyond the final solvent front. After chromatography, carrier spots were located under ultraviolet light and each chromatogram

lane was divided into three sections; (i) origin to nucleoside spot, (ii) nucleoside spot, and (iii) remainder to the solvent front (nucleotide area). These chromatogram sections, and another containing the unchromatographed sample, were inserted into counting vials which contained counting fluid (a toluene-fluor solution (120)) and were assayed for ^{14}C activity in a Nuclear-Chicago Model 725 liquid scintillation system. The amount of nucleotide formed per gram cells was calculated from the radioactivity (counts per minute, cpm) found in the nucleotide areas of chromatograms, the fraction of the PCA extract chromatographed, the weight of cells extracted and the specific activity (cpm per µmole) of the labelled base from which the nucleotide was derived. The latter was determined from radioactive measurements made on paper mounted base samples: measured volumes of labelled base solutions of known concentration were dried on the same type of paper used in chromatography and the radioactivity was assayed as described above. The channels ratio method (153) was employed routinely to detect "quenching"; it was found that quenching did not occur in these experiments.

In analyzing the "total nucleotides" derived from the labelled hypoxanthine, the same general procedure was used, however, solvent B was used in chromatography. With this solvent bases have a higher mobility than nucleosides, which move at a rate intermediate between bases and the slow-moving nucleotides. Purine nucleotides derived from

hypoxanthine are found between the origin and the inosine spot.

Radioactivities in the chromatogram sections totalled more than 90% of that in the unchromatographed samples. In extracts from tumor cells which had been treated in vivo with labelled base, approximately 90% of the chromatographed radioactivity was present in the nucleotide area.

Determination of individual nucleotides derived from 6MP or Me6MPR: DEAE-Sephadex chromatography (121) was used to isolate individual nucleotides for determination.

Samples (6 to 15 ml) of neutralized PCA extracts were applied to 1 x 90 cm columns of DEAE-Sephadex (acetate form); these columns were eluted with gradients of triethylammonium acetate and the eluate was collected in 5 ml fractions. For each eluate fraction the optical density at 260 mμ (OD_{260}) (or at 292 mμ for determination of Me6MPR phosphate) was measured; as well, the radioactivity of each fraction was assayed using Bray's counting fluid (122) and liquid scintillation counting. A sample (0.5 ml) of each fraction was mixed with 4.5 ml of Bray's counting fluid and counted in Nuclear-Chicago Mark I liquid scintillation system. Radioactive peaks in the elution profile were identified by their positions relative to OD_{260} peaks of naturally-occurring nucleotides (50). The amount of individual nucleotide formed per gram of cells was calculated from the total radioactivity in the radioactive peak, the fraction of the PCA extract chromatographed, the weight of

cells extracted, and the specific activity of radioactive substrate from which the nucleotide was derived. The specific activity was determined by mixing 0.5 ml of the diluted solution of the labelled substrate of known concentration with 4.5 ml Bray's solution and the radioactivity was assayed as described above. Again, quenching did not occur in these experiments.

Paper chromatography: Descending paper chromatography (with Whatman No. 40 paper) employed the following solvent systems:

Solvent A: isoamyl alcohol and aqueous 5% Na_2HPO_4, mutually saturated and used in equal volumes (123);

Solvent B: *n*-butanol-glacial acetic acid-water (60:15:25, v/v) (124).

D. Chemicals

Me6MPR-(methyl-^{14}C), prepared by reacting 6MPR with ^{14}C-methyl iodide (38), was kindly provided by Dr. I.C. Caldwell of this laboratory. Other radioactive compounds were commercial products.

6MP and other purine analogues were obtained from the Cancer Chemotherapy National Service Center, Bethesda, Md., or were commercial products.

III. RESULTS

A. Combination Chemotherapy

1. Combination treatment of EAC with 6MPR and Me6MPR

A derivative of 6MP, thioinosine (6MPR), inhibits a number of transplantable mouse tumors (125) and has a higher therapeutic index[8] than 6MP in the treatment of mice bearing Adenocarcinoma 755 (126). However, as shown in Table II, 6MPR was found to be ineffective against the Ehrlich ascites tumor; the survival time of tumor-bearing mice was lengthened to a minor extent only, even with dose levels as high as 150 mg/kg. The latter dosage, in terms of number of moles of 6MP contained therein, was approximately equivalent to the LD_{10} for 6MP[9]. After treatment of EAC-bearing mice with 6MP at 50% LD_{10}, 60% of mice were alive on the 50th day after implantation without apparent tumors, under the experimental conditions used in this work[7]. In the experiment of Table II, the highest dose of 6MPR was less than the reported LD_{10}[10], and toxic signs were not evident.

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[8] Ratio of the dose that kills 10% of the host animals (LD_{10}) to the dose that reduces tumor growth to 10% of control values.

[9] The LD_{10} of 6MP for Ha/ICR mice treated once daily for 5 days is about 80 mg/kg (M.C. Wang, unpublished results).

[10] The LD_{10} of 6MPR for Swiss mice treated once daily for 7 days is 249 mg/kg (127).

TABLE II

Failure of thioinosine to inhibit the growth of the Ehrlich ascites carcinoma

Dose (mg/kg)	Average survival (days)
Control (saline)	16.2 ± 4.6*(10)**
20	24.1 ± 3.5 (10)
40	21.9 ± 1.9 (10)
70	22.8 ± 3.0 (9)
100	21.7 ± 2.6 (10)
150	21.3 ± 3.6 (10)

NOTE: Mice weighing 20 to 30 grams were used.

* Mean Deviation.

** Number of mice.

It is known that 6MPR is converted in rats and dogs to 6-thiouric acid (125), which is therapeutically inactive (15,128). Presumably, this inactivation process involves the initial cleavage of 6MPR by purine nucleoside phosphorylase to release 6MP, which is then oxidized by xanthine oxidase to 6-thiouric acid. Since mouse tissues possess both purine nucleoside phosphorylase (29,129) and xanthine oxidase (16-18), conversion of 6MPR to 6-thiouric acid would be expected in this animal. In mice bearing EAC, the inactivation of 6MPR would occur mainly in the normal tissues and not in the tumor cells, because the latter have only very low xanthine oxidase activity[11]. This raises the possibility that the low therapeutic effect with 6MPR could be due to a rapid removal of this drug from the peritoneal cavity, with subsequent catabolism in normal tissues or excretion.

Because Me6MPR inhibited the cleavage of other purine nucleosides by intact EAC cells (111), it was considered possible that Me6MPR might reduce 6MPR catabolism; this reasoning, which in retrospect was naïve, led to a test of the therapeutic effect of combinations of 6MPR and Me6MPR against EAC. This combination was found to be much more effective than either agent alone; however, after further study, it became apparent that inhibition of 6MPR catabolism was not the reason for the therapeutic synergism

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[11] M.C. Wang, Master of Science thesis, The University of Alberta (1965).

resulting from combination of the drugs.

As shown in Table III, treatment of EAC-bearing mice with either 6MPR or Me6MPR at 10 mg/kg resulted in small increases in survival time (13% and 30%, respectively). However, treatment with a mixture of these drugs, each at the level of 10 mg/kg, had the result that half the animals were living with no apparent tumors, 100 days after implantation. Solid tumors, which caused death of 3 mice in this experiment, apparently originated from tumor cells deposited along needle paths during implantation.

It became apparent that the therapeutic synergism could not be attributed to the ability of Me6MPR to inhibit the catabolism of 6MPR because two other analogue nucleosides, 2-amino-6-chloropurine ribonucleoside (ACPR) or 2-amino-6-(o-chlorobenzylmercapto)purine ribonucleoside (CBTGR), which are as potent as Me6MPR in inhibiting nucleoside metabolism (112), failed to potentiate the antitumor acitivty of 6MPR. As may be seen in Table IV, concurrent treatment with 6MPR and Me6MPR (70 and 6 mg/kg, respectively) effectively inhibited the growth of EAC with the result that 6 out of 10 mice were tumor-free at 100 days. However, ACPR or CBTGR could not take the place of Me6MPR at the equivalent dosage, as may be seen in Table IV.

Since 6MPR is readily phosphorolysed by EAC cells (130) it was considered possible that the 6MP so released was the active component in the 6MPR-Me6MPR combination. The experiment of Table V affirmed this possibility, showing that

TABLE III

Synergism resulting from the concurrent administration of 6MPR and Me6MPR

Agent	Dose (mg/kg)	Average survival of mice dying with ascites tumors (days)	Number of 100 day survivors
Control (saline)		17.6 ± 3.2*(10)**	0
6MPR	10	19.9 ± 3.7 (10)	0
Me6MPR	10	22.4 ± 3.0 (10)	0
6MPR plus† Me6MPR	10 10	23.5 ± 2.5 (2)	5

* Mean deviation.

** Number of mice.

† 3 mice in this group died with solid tumors on the 42nd, 51st and 74th days after tumor implantation.

TABLE IV

Failure of two inhibitors of ribonucleoside metabolism to potentiate the therapeutic effect of 6MPR

Agent	Dose (mg/kg)	Average survival of mice dying with ascites tumors (days)	Number of 100 day survivors
Experiment A			
Control (saline)†		19.1 ± 2.1*(9)**	0
6MPR	70	26.6 ± 4.6 (10)	0
Me6MPR	6	24.2 ± 2.0 (10)	0
6MPR plus†† Me6MPR	70 6		6
Experiment B			
Control (saline)		19.5 ± 1.5 (10)	0
ACPR#	10	19.5 ± 6.4 (10)	0
6MPR plus ACPR	70 10	19.0 ± 3.0 (10)	0
Experiment C			
Control (saline)		15.6 ± 4.0 (10)	0
CBTGR##	8.3	13.0 ± 3.4 (10)	0
6MPR plus CBTGR	70 8.3	14.4 ± 4.0 (10)	0

* Mean deviation.

** Number of mice.

† One mouse in this group died with a solid tumor on the 72nd day after tumor implantation.

†† 4 mice in this group died with solid tumors on the 58th, 69th, 70th and 83rd days after tumor implantation.

2-amino-6-chloropurine ribonucleoside.

2-amino-6-(o-chlorobenzylmercapto)purine ribonucleoside.

TABLE V

Replacement of 6MPR by 6MP in 6MPR-Me6MPR combination therapy

Agent	Dose (mg/kg)	Average survival of mice dying with ascites tumors (days)	Number of 100 day survivors
Control (saline)		14.1 ± 3.7* (10)**	0
6MP	6	15.3 ± 4.4 (10)	0
6MPR	9	19.7 ± 3.3 (10)	0
Me6MPR	6	23.2 ± 2.0 (10)	0
6MP plus† Me6MPR	6	28 (1)	5
6MPR plus†† Me6MPR	9 6	61 ± 26 (2)	4

* Mean deviation.

** Number of mice.

† The other 4 mice in this group died with solid tumors on the 45th, 61st, 76th, and 80th days after tumor implantation.

†† The other 4 mice in this group died with solid tumors on the 49th, 63rd, 67th, and 69th days after tumor implantation.

6MP or 6MPR, at approximately equimolar amounts, synergized with Me6MPR in inhibition of the growth of EAC. As will be seen later, ETGRII tumor cells, which are resistant to the 6MP-Me6MPR combination (Table XI), are also resistant to the 6MPR-Me6MPR combination (Table XII).

2. Combination treatment of EAC with 6MP and Me6MPR

The therapeutic synergism produced by the combined use of 6MP and Me6MPR may also be demonstrated by measurement of the volume or mass of tumor cells remaining after therapy. Synergism was clearly demonstrated with this method when the dosage of drugs was low; after high drug doses the remaining tumor masses were small and consequently differences among the treatment results were less than the sensitivity of the method.

Table VI shows the results of two such experiments. It is seen that when ineffective dosages of 6MP were used together with Me6MPR, potentiation of growth inhibitory effects resulted. For example, in Experiment B, 6MP treatments produced no effect at doses that ranged from 0.5 to 4 mg/kg, and Me6MPR at 4 mg/kg caused the proliferation of tumor cells to lag 2.5 doublings behind the control; when the two agents were used together at these doses, the growth of the tumor cells lagged at least 5.7 doublings behind that of control cells. Thus, the effect produced by the combination treatment was clearly synergistic.

The synergism resulting from the combined use of 6MP and Me6MPR has been confirmed repeatedly. In all such

TABLE VI

Synergistic inhibition of Ehrlich ascites carcinoma by combined treatment with 6MP and Me6MPR

Agent	Dose (mg/kg)	Average mass of packed tumor cells per mouse (grams)	Number of tumor cell doublings†
Experiment A			
Control (saline)		1.85 ± 0.23*(30)**	6.5
6MP	40	0.17 ± 0.07 (10)	3.1
6MP	20	0.33 ± 0.06 (9)	4.0
6MP	10	0.69 ± 0.21 (10)	5.1
6MP	4	0.17 ± 0.14 (10)	5.9
Me6MPR	20	0.04 ± 0.01 (10)	1.0
Me6MPR	10	0.08 ± 0.04 (10)	2.0
Me6MPR	5	0.08 ± 0.03 (10)	2.0
Me6MPR	2	0.68 ± 0.34 (10)	5.1
6MP plus Me6MPR	20 10	0.02 ± 0.01 (10)	0
6MP plus Me6MPR	10 5	0.03 ± 0.01 (10)	1.6
6MP plus Me6MPR	4 2	0.15 ± 0.13 (10)	2.9
6MP plus Me6MPR	0.8 2	0.27 ± 0.18 (10)	3.8

(Continued)

TABLE VI (Continued)

Agent	Dose (mg/kg)	Average volume of packed tumor cells per mouse (ml)	Number of tumor cell doublings†
Experiment B			
Control (saline)		3.04 ± 0.47 (10)	7.3
6MP	4	3.02 ± 0.65 (9)	7.2
6MP	2	3.38 ± 0.62 (10)	7.4
6MP	1	4.07 ± 0.60 (10)	7.7
6MP	0.5	3.41 ± 0.64 (10)	7.4
Me6MPR	4	0.53 ± 0.29 (10)	4.7
6MP plus Me6MPR	4 4	0.02 ± 0.02 (10)	0
6MP plus Me6MPR	2 4	0.02 ± 0.01 (10)	0
6MP plus Me6MPR	1 4	0.05 ± 0.03 (10)	1.3
6MP plus Me6MPR	0.5 4	0.06 ± 0.04 (8)	1.6

NOTE: The mass of tumor cells (Experiment A) was measured on the 7th day after implantation as described in MATERIALS AND METHODS. The volume of packed tumor cells (Experiment B) was measured on the 10th day after implantation.

* Mean deviation.

** Number of mice.

† Calculated by the method of Finney _et al_. (131).

experiments the administration of drugs was started 24 hours after inoculation of tumor. To determine whether the combination treatment was also effective against the tumor in more advanced stages, treatment was initiated several days after implantation. Table VII shows the results of the combination treatment begun 4 days after implantation of EAC. As can be seen, the combination of 6MP and Me6MPR, at 32 and 20 mg/kg respectively, was toxic and killed 9 out of 10 mice. However, following treatment with a combination of 6MP and Me6MPR at one half of these doses, 9 out of 10 mice were alive and without evident tumor on the 50th day after implantation. Treatments with individual drugs alone at the same doses produced a much smaller therapeutic effect. Thus, the combination treatment produced distinct synergism even though the treatment was started 4 days after inoculation.

When treatments were started on the 7th day after the tumor implantation, the synergism was still seen, but was less effective. It is shown in Table VIII that treatment of the tumor-bearing mice with Me6MPR at 10 mg/kg, or with 6MP at 16 to 64 mg/kg, increased survival time only slightly, but when these doses of 6MP and Me6MPR were used together, a number of long-term survivors resulted. The high toxicity of Me6MPR and of the 6MP-Me6MPR combination toward the EAC-bearing host was much in evidence in this experiment. All animals were distended with ascitic fluid when treatment was begun in this experiment. Effective treatments caused rapid

TABLE VII

Combination treatment with 6MP and Me6MPR begun 4 days after implantation of the Ehrlich ascites carcinoma

Agent	Dose (mg/kg)	Average survival of mice dying with ascites tumor (days)	Number of 50 day survivors
Control (saline)		16.4 ± 2.1*(18)**	2
6MP	32	22.7 ± 8.8 (7)	3
6MP	16	29.6 ± 12.4 (8)	2
Me6MPR†	40	28 (1)	0
Me6MPR	20	40.5 ± 6.0 (4)	4
Me6MPR	10	25.9 ± 2.7 (8)	2
6MP plus† Me6MPR	32 20	29 (1)	0
6MP plus Me6MPR	16 10	47 (1)	9

NOTE: None of the mice in this experiment died with solid tumors.

* Mean deviation.

** Number of mice.

† 9 mice in this group died of toxicity without apparent tumor; these mice died early and characteristically had ruffled fur, hunched posture, weight loss, and sometimes diarrhea before death.

disappearance of this distension, with some animals going on to indefinite survival and others to toxic deaths. This experiment has been repeated with similar results.

In the experiment of Table VIII, treatment with the combination of 6MP and Me6MPR at 16 and 10 mg/kg, respectively, was superior to other treatments, including treatment with 6MP or Me6MPR alone. These data indicate that the drug combination can elicit stronger antitumor effects within the limit of tolerable toxicity than the individual drugs used alone. It should be noted that Me6MPR produced a very strong toxic effect at dosages above 40 mg/kg in tumor-bearing mice, even though the LD_{10} in tumor-free mice was approximately 50 mg/kg[12]. It is possible that a rapid destruction of tumor cells by high dosages of Me6MPR led to the release of a large amount of toxic substances, or that the stress imposed by the tumor rendered mice more sensitive to Me6MPR.

3. Biochemical transformations of drugs required for the manifestation of synergism

As a preliminary step to exploration of the biochemical mechanism of the 6MP-Me6MPR synergism, drug transformations that were obligatory in the synergism were investigated.

Cleavage of Me6MPR is not involved in the synergism: Me6MPR is cleaved phosphorolytically only to a small extent (29,38,40,69); hence, the formation of Me6MP is not likely to be involved in the synergism. Table IX shows that treat-

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[12] M.C. Wang, unpublished results.

TABLE VIII

Combination treatment with 6MP and Me6MPR begun 7 days after implantation of Ehrlich ascites carcinoma

Agent	Dose (mg/kg)	Average survival of mice dying with ascites tumors (days)	Survival of individual mice dying with solid tumors (days)	Number of 50 day survivors
Control	(saline)	19.9 ± 5.3* (40)**		2
6MP	80	16.8 ± 1.8 (10)		0
6MP	64	17.2 ± 2.4 (10)		0
6MP	48	20.2 ± 2.0 (10)		0
6MP	32	16.7 ± 1.7 (9)		1
6MP	16	22.5 ± 4.6 (8)	45	1
Me6MPR†	50	12.0 ± 1.0 (2)		0
Me6MPR†	40	10.2 ± 1.0 (2)		0
Me6MPR†	30	24.9 ± 4.4 (7)		2
Me6MPR	20	24.7 ± 3.0 (8)		2
Me6MPR	10	22.1 ± 3.7 (10)		0
6MP plus† Me6MPR	64 10	27.9 ± 9.3 (7)		2
6MP plus Me6MPR	48 10	17.2 ± 3.4 (5)		4
6MP plus Me6MPR	32 10	19.9 ± 0.6 (8)		2
6MP plus Me6MPR	16 10	17.3 ± 3.1 (4)	49,50	4

* Mean deviation.
** Number of mice.
† Other mice in this group died of toxicity without apparent tumors (see footnote of Table VII).

TABLE IX

Failure of Me6MP to potentiate the antitumor activities of 6MP and 6MPR

Agent	Dose (mg/kg)	Average survival of mice dying with ascites tumors (days)	Number of 50 day survivors
Experiment A			
Control (saline)		16.6 ± 2.1*(9)**	1
6MP†	10	17.4 ± 2.4 (9)	0
Me6MP	10	21.7 ± 6.7 (10)	0
Me6MP	20	21.4 ± 4.1 (10)	0
Me6MP	40	17.7 ± 2.7 (10)	0
Me6MP	80	20.1 ± 1.7 (9)	1
6MP plus Me6MP	10 10	23.9 ± 2.1 (10)	0
6MP plus†† Me6MP	10 20	22.4 ± 3.4 (9)	0
6MP plus Me6MP	10 40	22.6 ± 6.2 (10)	0
6MP plus Me6MP	10 80	21.8 ± 3.0 (10)	0
Experiment B			
Control (saline)		18.0 ± 4.4 (10)	0
6MPR	20	15.7 ± 3.6 (10)	0
Me6MP	10	19.2 ± 4.2 (10)	0
Me6MP	20	19.4 ± 4.3 (10)	0
Me6MP	40	20.4 ± 4.5 (10)	0
6MPR plus Me6MP	20 10	18.2 ± 3.1 (10)	0

(Continued)

TABLE IX (Continued)

Agent	Dose (mg/kg)	Average survival of mice dying with ascites tumor (days)	Number of 50 day survivors
6MPR plus Me6MP	20 20	23.3 ± 3.6 (10)	0
6MPR plus Me6MP	20 40	22.6 ± 1.4 (10)	0

* Mean deviation.

** Number of mice.

† One mouse in this group died with a solid tumor on the 39th day after tumor implantation.

†† One mouse in this group died with a solid tumor on the 42nd day after tumor implantation.

ment with 6MP plus Me6MP (Experiment A), or with 6MPR plus Me6MP (Experiment B), did not result in extension of the survival time of EAC-bearing mice beyond that of controls. From these results it is concluded that release of Me6MP is not involved in the therapeutic effect of Me6MPR, or in the synergism with 6MP.

Formation of drug nucleotides as an essential step in the 6MP-Me6MPR synergism: It has been shown clearly that formation of nucleotide derivatives of 6MP and Me6MPR is an essential step in the individual antitumor activities of these two agents (37,63,99,100). Consequently, it was considered likely that these transformations were also obligatory for the synergism.

To examine this question, combination therapy with the 6MP-Me6MPR pair was tested against several thiopurine-resistant tumors which were known to have defects in the conversion of 6MP or Me6MPR to nucleotides. Table X summarizes the abilities of the resistant tumor cells to synthesize these nucleotide derivatives (other information about these tumors is found in Table I). As can be seen, the resistant cells, with the exception of EAC-R2 cells, phosphorylate Me6MPR at rates comparable to that of the parent line, EAC; EAC-R2 tumor cells are also exceptional in this group in that their capacity to synthesize 6MPRP from a test dose of 6MP is similar to that of EAC.

The results of combination chemotherapy of these resistant tumors are shown in Table XI. In all of these experi-

TABLE X

Formation of analogue nucleotides in thiopurine-resistant sublines of EAC

Tumor	Rate of nucleotide formation ($m\mu$moles per gram cells per minute)	
	Me6MPRP *in vitro* assay (38)	6MPRP *in vivo* assay (54)
EAC	175	4.3
EAC-R1	183	<0.1
EAC-R2	<10	3.7
ETGRI	161	1.3
ETGRII	124	None

NOTE: To measure the capacity of tumor cells to synthesize Me6MPR phosphate, tumor cells (0.2 gram per ml) were incubated with 0.5 m*M* Me6MPR-(methyl-^{14}C) and the PCA extracts of tumor cells, taken from incubation mixtures at 1 minute intervals, were analyzed for Me6MPR phosphate by the paper chromatographic method (38).

To estimate the rate of 6MPRP formation, each of 5 tumor-bearing mice received by ip injection 5 μmoles of non-radioactive 6MP; the PCA extracts of tumor cells collected 30 minutes later were analyzed for 6MPRP by ion exchange chromatography (54).

TABLE XI

Combination therapy of thiopurine-resistant tumors

Agent	Dose (mg/kg)	Average survival of mice (days)	Number of 50 day survivors
Experiment A: EAC-R1			
Control (saline)		15.3 ± 2.7*(30)**	0
6MP	64	9.8 ± 1.2 (10)	0
6MP	32	9.7 ± 1.5 (10)	0
6MP	16	11.3 ± 1.2 (10)	0
Me6MPR	40	9.8 ± 0.5 (10)	0
Me6MPR	20	13.0 ± 3.8 (10)	0
Me6MPR	10	14.3 ± 2.2 (10)	0
6MP plus Me6MPR	32 20	9.4 ± 1.3 (10)	0
6MP plus Me6MPR	16 10	13.9 ± 3.1 (10)	0
Experiment B: ETGRI			
Control (saline)		11.2 ± 2.5 (35)	0
6MP	80	9.7 ± 0.9 (9)	0
6MP	64	8.2 ± 1.1 (9)	0
6MP	48	8.1 ± 0.7 (10)	0
6MP	32	9.2 ± 1.0 (10)	0
6MP	16	9.9 ± 1.9 (9)	1
Me6MPR	50	13.8 ± 6.9 (10)	0
Me6MPR	40	10.0 ± 1.0 (10)	0
Me6MPR	30	13.9 ± 0.2 (9)	1
Me6MPR	20	15.4 ± 2.2 (10)	0
Me6MPR	10	17.4 ± 1.9 (10)	0
6MP plus Me6MPR	32 20	11.6 ± 0.9 (9)	1

(Continued)

TABLE XI (Continued)

Agent	Dose (mg/kg)	Average survival of mice (days)	Number of 50 day survivors
Experiment C: ETGRI			
Control (saline)		10.3 ± 2.5 (29)	0
6MP	20	9.3 ± 2.9 (10)	0
6MP	10	9.4 ± 1.7 (10)	0
6MP	5	11.7 ± 2.7 (10)	0
Me6MPR	10	15.7 ± 2.4 (9)	1
6MP plus Me6MPR	20 10	19.0 ± 6.3 (9)	1
6MP plus Me6MPR	10 10	16.9 ± 3.4 (10)	0
6MP plus Me6MPR	5 10	20.7 ± 6.3 (10)	0
Experiment D: ETGRII			
Control (saline)		15.6 ± 2.9 (25)	0
6MP	10	18.6 ± 2.7 (9)	1
6MP	5	15.8 ± 2.4 (10)	0
Me6MPR	10	22.0 ± 4.0 (10)	0
6MP plus Me6MPR	10 10	19.3 ± 5.5 (10)	0
6MP plus Me6MPR	10 5	23.8 ± 2.6 (10)	0

(Continued)

TABLE XI (Continued)

Agent	Dose (mg/kg)	Average survival of mice (days)	Number of 50 day survivors
Experiment E: ETRGII			
Control (saline)		15.7 ± 2.0 (35)	0
6MP	80	9.8 ± 1.5 (10)	0
6MP	64	10.2 ± 2.5 (10)	0
6MP	48	10.2 ± 1.6 (10)	0
6MP	32	11.3 ± 2.4 (10)	0
6MP	16	17.0 ± 4.2 (10)	0
Me6MPR	50	15.5 ± 5.8 (10)	0
ME6MPR	40	17.8 ± 7.4 (10)	0
Me6MPR	30	17.5 ± 2.0 (10)	0
Me6MPR	20	19.2 ± 1.6 (10)	0
Me6MPR	10	20.0 ± 2.4 (10)	0
6MP plus Me6MPR	32 20	11.8 ± 2.1 (10)	0
6MP plus Me6MPR	16 10	17.9 ± 5.9 (10)	0
Experiment F: EAC-R2			
Control (saline)		14.1 ± 2.5 (28)	0
6MP	64	10.7 ± 1.1 (10)	0
6MP	32	20.5 ± 2.6 (10)	0
6MP	16	23.4 ± 6.3 (10)	0
Me6MPR	40	7.5 ± 1.0 (10)	0
Me6MPR	20	7.6 ± 0.7 (10)	0

(Continued)

TABLE XI (Continued)

Agent	Dose (mg/kg)	Average survival of mice (days)	Number of 50 day survivors
6MP plus Me6MPR	32 20	11.4 ± 1.5 (10)	0
6MP plus Me6MPR	16 10	14.4 ± 2.1 (10)	0

NOTE: When EAC-bearing mice were treated with these drugs using the treatment schedule employed in these experiments, 60% of the mice which had been treated with 32 mg/kg of 6MP and 30% of those which had received Me6MPR at 20 mg/kg were alive 50 days after tumor implantation. Combination treatment with 6MP and Me6MPR at one-half of these doses had the result that 80% of mice were alive and apparently tumor-free on the 50th day after implantation.

* Mean deviation.

** Number of mice.

ments, treatments were started 24 hours after tumor implantation and were given once daily for a total of 5 days.

As can be seen, the resistant EAC sublines, EAC-R1, ETGRI, and ETGRII were found to be resistant to combination therapy (Experiments A to E). As shown in Table X, these tumor cells have a decreased ability to synthesize 6MP nucleotide, but have retained ability to phosphorylate Me6MPR. Thus failure to form 6MP nucleotide is associated with resistance to 6MP and to the agents in combination. Schabel et al. (7) also showed that 6MP-Me6MPR combinations failed to produce synergistic effects against a 6MP-resistant subline of mouse leukemia L1210, which lacked hypoxanthine-guanine phosphoribosyltransferase.

It is also seen in Table XI that the EAC-R2 tumor, which was selected for resistance to Me6MPR (63), was resistant to combination therapy (Experiment F). This tumor phosphorylates only trace amounts of Me6MPR, but has retained the ability to synthesize 6MP nucleotide (Table X); therefore the resistance of this tumor to combination therapy suggests that phosphorylation of Me6MPR is also an essential part of the mechanism of synergism.

Because ETGRII tumor cells were known to phosphorylate 6MPR (34) and Me6MPR (see Table X), it was considered possible that this tumor would respond to combination treatment with these two analogue nucleosides. However, it was found that these two agents did not synergistically inhibit the growth of ETGRII. As shown in Table XII, the tumor-bearing

TABLE XII

Combination therapy of ETGRII with 6MP and Me6MPR

Agent	Dose (mg/kg)	Average survival of mice dying with ascites tumor (days)	Number of 50 day survivors
Control (saline)		16.2 ± 1.7*(28)**	0
6MPR	376	16.1 ± 2.3 (9)	1
6MPR	188	16.6 ± 3.0 (9)	1
6MPR	94	16.7 ± 3.6 (10)	0
Me6MPR	40	18.0 ± 4.0 (9)	1
Me6MPR	20	22.8 ± 3.8 (10)	0
Me6MPR	10	20.8 ± 2.6 (10)	0
6MPR plus Me6MPR	188 20	12.8 ± 3.7 (10)	0
6MPR plus Me6MPR	94 10	22.2 ± 1.1 (9)	1

* Mean deviation.

** Number of mice.

mice treated with 6MPR-Me6MPR combination, at dosages of 188 and 20 mg/kg, respectively, died earlier than untreated mice. Combination therapy at half the above dosage was not better than Me6MPR alone. It was subsequently found that 6MP nucleotide was formed from 6MPR in ETGRII cells at low rates only[13]. Thus, the observed resistance of ETGRII to combination therapy with the 6MPR-Me6MPR pair does not contradict the idea that the formation of nucleotides from both 6MP and Me6MPR was necessary for synergy.

B. Metabolism of 6MP and Me6MPR in Ehrlich Ascites Tumor Cells

1. Influence of one drug upon the metabolism of the other

It was considered possible that the therapeutic potentiation seen in these experiments might have an explanation in the influence of one member of the drug pair on the metabolism of the other; for example, the intracellular pools of drug nucleotides ("active" forms) might be changed in size, or the kinetics of their decline might be altered. This possibility was tested by determining whether treatment of EAC cells *in vivo* with 6MP (or Me6MPR) 30 minutes prior to a test dose of Me6MPR (or 6MP) influenced conversion of the latter into the nucleotide form. It is seen in Table XIII

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[13] Mice bearing 7-day ETGRII tumors were injected ip with 18 μmoles 6MPR each; one hour later the tumor cells contained 0.008 μmoles 6MPRP per gram. In a similar experiment (Figure 4), mice with the Ehrlich ascites carcinoma were each injected with 2.5 μmoles of 6MP; one hour later these tumor cells contained 0.040 μmoles 6MPRP per gram.

that 6MP had no effect on the phosphorylation of Me6MPR, but exposure of the tumor cells to Me6MPR doubled the amount of 6MPRP synthesized. When 6MP and Me6MPR were injected simultaneously the enhancement of 6MP anabolism was absent (Table XIV). This result seemed to argue against altered drug metabolism as a mechanism of the synergism because in therapy the drugs are given together. However, it was realized that one drug treatment might influence the tumor cell's metabolism of the drugs given in the next treatment, 24 hours later. The experiment of Table XV examined this possibility, using drug dosages known to have therapeutic effects. It is seen that when 6MP was given in two successive treatments 24 hours apart, nucleotide formation was stimulated about 3-fold by Me6MPR. In a similar experiment, summarized in Table XVI, it was shown that Me6MPR phosphorylation in EAC cells *in vivo* was not enhanced by 6MP.

The effects of prior treatment on 6MP anabolism in thiopurine-resistant tumor cells were examined in the experiments of Table XVII, which were similar to those of Table XIV. Again, the experimental design was intended to determine whether one therapeutic treatment with the 6MP-Me6MPR pair would influence the metabolism of 6MP in the next following treatment. In contrast with EAC results, nucleotide synthesis from 6MP was not significantly enhanced by Me6MPR in cells of the EAC-Rl, EAC-R2, and ETGRII sublines. In ETGRII cells, 6MP anabolism was stimulated 5-fold by Me6MPR, although the total amount of nucleotides formed was

TABLE XIII

Enhancement of 6MPRP formation in EAC cells by pretreatment with Me6MPR

		Concentration (mμmoles per gram cells)	
Pretreatment	Substrate	6MPRP	Me6MPRP
Experiment A			
None	6MP-8-^{14}C	35	8
Me6MPR	6MP-8-^{14}C	74	11
Experiment B			
None	Me6MPR		270
6MP	Me6MPR		290

NOTE: In experiment A, 1.2 μmoles of Me6MPR was injected into the ascitic fluid of each of 3 mice bearing EAC (7 days after implantation); 30 minutes later these mice received 3 μmoles each of 6MP-8-^{14}C by the same route, as did a control group of EAC-bearing mice which were not pretreated. The mice were killed 2 hours later and the pooled tumor cells from each group were analyzed for labelled 6MPRP and Me6MPRP by DEAE-Sephadex chromatography (121).

In Experiment B, the same procedure was followed, except that 6MP (3 μmoles per mouse) was injected 30 minutes before Me6MPR (1 μmole per mouse). OD measurements at 292 mμ were used to locate and determine Me6MPRP in chromatographic column eluates.

TABLE XIV

Failure of Me6MPR to stimulate nucleotide synthesis from 6MP in EAC cells when these drugs are injected simultaneously

Time after test dose (hours)	Total nucleotide-^{14}C (mμmoles per gram cells)	
	Control	Me6MPR
1	101	62
2	43	32
12	13	11

NOTE: On the 7th day after implantation with EAC, mice were injected ip with 3 μmoles 6MP-8-^{14}C (control), or with 3 μmoles 6MP-8-^{14}C plus 1.2 moles Me6MPR. At the indicated times, 3 mice were withdrawn from each group and their ascitic fluids pooled. Tumor cells were spun down at 2,400 x g for 10 minutes and extracted with perchloric acid for the determination of ^{14}C in the nucleotide fraction, as described in MATERIALS AND METHODS.

TABLE XV

Effect of prior therapy on the synthesis of nucleotide from 6MP in EAC cells

First injection	Second injection	Total nucleotide-^{14}C (mμmoles per gram cells)
6MP-8-^{14}C	6MP-8-^{14}C	170
6MP-8-^{14}C plus Me6MPR	6MP-8-^{14}C plus Me6MPR	468

NOTE: EAC-bearing mice were used on the 6th day after tumor implantation. Two mice received ip 3 μmoles of 6MP-8-^{14}C each and this was repeated 24 hours later; two other mice received the same dosage of 6MP-8-^{14}C, together with 1.2 μmoles Me6MPR and this was also repeated 24 hours later. Two hours after the last injection, the tumor from each mouse was collected separately and for each the total labelled nucleotide was determined. The data above are the averaged values for each pair.

TABLE XVI

Effect of prior therapy on the phosphorylation of Me6MPR in EAC cells

First injection	Second injection	Me6MPR phosphate (mμmoles per gram cells)	
		Total*	Labelled
Me6MPR	Me6MPR-(methyl-^{14}C)	450	244
Me6MPR plus 6MP	Me6MPR-(methyl-^{14}C) plus 6MP	485	283

NOTE: EAC-bearing mice were used on the 6th day after tumor implantation. Two mice were injected ip with non-radioactive Me6MPR (1.2 μmoles per mouse) and then with ^{14}C-labelled Me6MPR (1.2 μmoles per mouse) 24 hours later. Two other mice received the same treatment, but in addition, each of these received 3 μmoles 6MP in each injection. Two hours after the last injection, the tumor cells from each group were pooled and analyzed for Me6MPRP by DEAE-Sephadex chromatography (121).

* Total Me6MPRP was estimated by the measurement of absorbance at 292 mμ; this represents Me6MPR phosphate derived from both injections of Me6MPR because intracellular pools of Me6MPR phosphate persist in EAC cells for long periods (see Table XIX).

TABLE XVII

Effect of prior treatment on the synthesis of nucleotide from 6MP in thiopurine-resistant sublines of EAC

Tumors	First injection	Second injection	Total nucleotide-^{14}C (mμmoles per gram cells)
EAC	6MP	6MP-8-^{14}C	102
	Me6MPR	6MP-8-^{14}C	494
	6MP plus Me6MPR	6MP-8-^{14}C plus Me6MPR	671
EAC-R1	6MP	6MP-8-^{14}C	11
	6MP plus Me6MPR	6MP-8-^{14}C plus Me6MPR	15
EAC-R2	6MP	6MP-8-^{14}C	127
	6MP plus Me6MPR	6MP-8-^{14}C plus Me6MPR	185
ETGRI	6MP	6MP-8-^{14}C	44
	6MP plus Me6MPR	6MP-8-^{14}C plus Me6MPR	202
ETGRII	6MP	6MP-8-^{14}C	3
	6MP plus Me6MPR	6MP-8-^{14}C plus Me6MPR	3

NOTE: Procedures were similar to those described in Table XV.

only one-third of that in EAC cells. It is appreciated that the drug nucleotide concentrations obtained in this experiment refer only to the 2 hour time point; however, the results are consistent with the chemotherapy experiments. These results agree with the conclusion reached above, that drug nucleotide formation is a necessary step in the therapeutic potentiation.

It is apparent in Tables VIII-XVII that the stimulatory effect of Me6MPR on 6MP anabolism is time-dependent: no stimulation was seen when the tumor cells were exposed to both 6MP and Me6MPR simultaneously, but exposure to Me6MPR 24 hours previously enhanced nucleotide formation from 6MP by several fold. To further study the nature of this Me6MPR effect, ^{14}C-labelled 6MP was injected into EAC-bearing mice at various times after injection of Me6MPR and the amounts of labelled nucleotides formed in the tumor cells were measured. The results are shown in Table XVIII. As can be seen in Experiment A, simultaneous injection of 6MP and Me6MPR did not promote nucleotide synthesis from 6MP; however, when Me6MPR injection preceded the test dose of 6MP-8-^{14}C by as little as 30 minutes, enhancement took place. In Experiment B the stimulatory effect of Me6MPR persisted for 48 hours and a maximal stimulation of 10-fold was achieved. In Experiment C it is seen that the stimulatory effect of Me6MPR increased rapidly with time and approached a maximum between 6 to 12 hours after injection, then slowly decreased. It is especially noteworthy that this effect was

TABLE XVIII

Persistence of the Me6MPR-pretreatment effect in EAC cells *in vivo*

Time interval between Me6MPR-pretreatment and injection of 6MP-8-^{14}C (hours)	Total nucleotide-^{14}C (mμmoles per gram cells)
Experiment A	
Control (without Me6MPR-pretreatment)	56
0	59
0.5	89
1	117
2	101
Experiment B	
Control (without Me6MPR-pretreatment)	87
6	660
12	883
24	726
48	708
Experiment C	
Control (without Me6MPR-pretreatment)	77
1.5*	119
3	291
6*	504
12	491
24	440
48*	435
96*	350

(Continued)

TABLE XVIII (Continued)

NOTE: On the 7th day after tumor implantation, groups of 3 mice (Expt. A) or pairs (Expts. B and C) received ip injections of 6MP-8-^{14}C (3 μmoles per mouse in Expts. A and B, or 2.5 μmoles in Expt. C); with the exception of control mice, these had been pretreated with Me6MPR at the indicated time intervals (1.2 μmoles per mouse). Two hours after injection of labelled 6MP, mice were killed, their ascitic fluids pooled and the tumor cells therefrom were extracted with perchloric acid. These extracts were neutralized and samples were analyzed for total nucleotide formation from 6MP by the paper chromatographic method. The remainders of certain extracts (*) in Expt. C were chromatographed on DEAE-Sephadex and Me6MPRP was determined by OD_{292} measurement; these results are reported in Table XIX.

long-lasting: 96 hours after the single injection of Me6MPR, the rate of nucleotide synthesis from 6MP was elevated 5-fold.

2. Mechanism of enhancement of 6MP anabolism

The persistence of Me6MPR phosphate in EAC cells *in vivo*: Earlier reports from this laboratory had noted that pools of Me6MPR phosphate, once formed in EAC cells, remained with little change for periods up to 24 hours (152). This unusual stability was thought likely to be the basis of the long-lived Me6MPR pretreatment effect. This idea was tested by determining the Me6MPR phosphate content of the tumor cells of Experiment C, Table XVIII. It was found that the concentration of this compound was maximal 3.5 hours after injection (Table XIX) and that substantial amounts of the analogue nucleotide were present in the tumor cells even after 98 hours. Thus, the slow rate of disappearance of Me6MPR phosphate from the tumor cells and the persistence of the stimulatory effect of Me6MPR appear to be related. As will be seen below, Me6MPR did not enhance 6MP anabolism in tumor cells which were unable to phosphorylate Me6MPR, a finding in agreement with the above suggestion.

Concentration of hypoxanthine-guanine phosphoribosyltransferase in Me6MPR-treated EAC cells: One possible basis for the relationship between Me6MPR phosphate and an elevated 6MP anabolism is that Me6MPR phosphate might increase the amount of the enzyme responsible for conversion of 6MP to nucleotide. Such a mechanism is unlikely, judging from the

TABLE XIX

Persistence of Me6MPRP in EAC *in vivo*

Time after injection of Me6MPR* (hours)	Me6MPRP (mμmoles per gram cells)
3.5	280
8	268
50	256
98	155

NOTE: This experiment is described in Table XVIII.

* Since tumor cells were collected 2 hours after injection of 6MP-8-^{14}C in the experiment of Table XVIII, the intervals listed above are 2 hours longer than those in Experiment C, Table XVIII.

data of Table XX, which indicate that Me6MPR pretreatment did not alter the activity of tumor cells extracts in the PRPP-supported synthesis of 6MP ribonucleotide.

Increased availability of PRPP as a cause of enhanced 6MP anabolism: Because Me6MPR 5'-phosphate is a potent inhibitor of glutamine-PRPP amidotransferase, it was considered possible that Me6MPR treatment might enhance 6MP anabolism by diverting PRPP away from purine nucleotide synthesis and into the phosphoribosyltransferase reaction. Thus, Me6MPR treatment might lead to the accumulation of PRPP in EAC cells. To test this possibility, PRPP concentrations in EAC cells *in vivo* were measured 6 hours after injection of Me6MPR. The results presented in Table XXI show that PRPP levels in the tumor cells were elevated 5- to 14-fold by exposure to Me6MPR.

It was apparent in Table XVII that 6MP anabolism in EAC-R2 tumor cells was stimulated by prior treatment with the 6MP-Me6MPR drug pair; in a similar experiment (data not shown) the same result was found for treatment with Me6MPR alone, as might be expected. Because EAC-R2 tumor cells are virtually unable to phosphorylate Me6MPR (see Table X), one would not expect Me6MPR treatment to increase PRPP concentrations in these cells, if the above arguments are valid; this result was found in Experiment D, Table XXI.

If diversion of PRPP away from purine nucleotide synthesis *de novo* is the cause of the Me6MPR enhancement of nucleotide synthesis from 6MP, it would be expected that

TABLE XX

Hypoxanthine-guanine phosphoribosyltransferase activity in Me6MPR-treated EAC cells

Source of extract*	Enzyme activity in the extract (mμmoles 6MPRP formed per minute per ml extract)
Saline-treated cells	54
Me6MPR-treated cells	57

NOTE: Incubation mixtures (100 μl) contained 50 μl of 10-fold diluted tumor extract and the following (μmoles): 6MP-8-^{14}C(0.1), PRPP(0.1), versene (0.1), $MgSO_4$(0.1), and pH 7.4 Tris-HCl buffer (5.0). After incubation at 37°C for 5 minutes, the reaction was terminated by adding 20 μl of 4 M formic acid. The formic acid mixtures (100 μl) were applied on paper, together with 6MPR and 6MPRP carriers, and chromatographed in solvent A. The amount of 6MPRP formed was calculated from the specific activity of the 6MP-8-^{14}C substrate and the radioactivity found in the 6MPRP carrier spot.

* Preparation of tumor extract: Three EAC-bearing mice were each injected ip with 1.2 μmoles Me6MPR and after 6 hours ascitic fluids were pooled and centrifuged at 2,400 x g for 10 minutes. The packed cells were then suspended in 0.2 M Tris-HCl buffer, pH 7.4, (0.2 gram cells per ml buffer) and disrupted with a sonic oscillator (29). The sonicate was then centrifuged at 33,000 x g for 1 hour and the supernatant fraction diluted 10-fold with the Tris-HCl buffer. The control extract was prepared in the same way using tumor cells from mice treated with saline instead of Me6MPR.

TABLE XXI

Effect of Me6MPR treatment on the PRPP content of ascites tumor cells

		PRPP concentrations (mμmoles per gram cells)	
Experiment	Tumor	In control cells	In Me6MPR-treated cells
A	EAC	42 ± 12*	208 ± 78
B	EAC	22 ± 20	314 ± 98
C**	EAC	13 ± 2	123 ± 43
D	EAC-R2	48 ± 18	33 ± 5

NOTE: Me6MPR (1.2 μmoles per mouse) was injected into the ascitic fluids of 4 mice bearing EAC and of 3 mice bearing EAC-R2 on the 7th day after tumor implantation; similar groups were injected with saline to serve as controls. Mice were killed 6 hours after injection of Me6MPR, and PRPP concentrations in tumor cells of each mouse were determined by the method of Henderson and Khoo (132).

* Mean deviation.

** The heated extracts of tumor cells were treated with charcoal (Norit A, acid washed) to remove purine and pyrimidine compounds before PRPP concentrations were assayed.

other reactions that utilize PRPP would also be enhanced. For this reason the synthesis of nucleotides from adenine, guanine, and hypoxanthine in Me6MPR-treated EAC cells was compared with that in untreated cells. The results summarized in Table XXII show that Me6MPR did stimulate these reactions distinctly.

If an increased availability of PRPP is the cause of enhanced 6MP anabolism, then it would be expected that elevated concentrations of PRPP would be found as long as the stimulatory effect of Me6MPR persists. Table XXIII shows that up to 96 hours after injection of Me6MPR, PRPP levels were still elevated. At this time point, nucleotide synthesis from 6MP was stimulated 5-fold by Me6MPR (see Table XVIII).

The idea that enhancement of 6MP anabolism by Me6MPR is due to the increase in availability of PRPP was further supported by showing that adenine interfered with the Me6MPR effect. AMP formation by the adenine phosphoribosyltransferase reaction can be expected to compete for available PRPP with 6MP ribonucleotide synthesis. Adenine would not interfere as a competitive substrate in the latter reaction and adenine nucleotides inhibit only to a slight extent the responsible enzyme, hypoxanthine-guanine phosphoribosyltransferase (133,154). It is seen in Table XXIV that pretreatment of EAC with Me6MPR stimulates the formation of nucletide from 6MP about 6-fold and that this stimulatory effect of Me6MPR was abolished when adenine was injected together with

TABLE XXII

Stimulation of nucleotide synthesis from purine bases by Me6MPR pretreatment of EAC cells

Bases	Nucleotide-^{14}C (mμmoles per gram cells) Untreated cells	Me6MPR-treated cells
Experiment A		
Adenine-8-^{14}C	304	509
Hypoxanthine-8-^{14}C	79	254
Experiment B		
Adenine-8-^{14}C	330	627
Guanine-8-^{14}C	113	296
Experiment C		
Hypoxanthine-8-^{14}C	87	224

NOTE: Me6MPR (1.2 μmoles per mouse) was injected into the ascitic fluids of EAC-bearing mice and after 6 hours (Expts. A and C) or 24 hours (Expt. B) labelled purines (3 μmoles per mouse, 2 mice for each base) were injected into the ascitic fluids, as specified above. After 30 minutes (Expts. B and C) or 2 hours (Expt. A) mice were killed, ascitic fluids from each pair were pooled and the tumor cells were extracted with PCA. The total labelled nucleotide fraction was determined by the paper chromatographic assay, as described in MATERIALS AND METHODS.

TABLE XXIII

Duration of the Me6MPR pretreatment effect on PRPP levels in EAC cells *in vivo*

Time after injection of Me6MPR* (hours)	PRPP (mμmoles per gram cells)
Without Me6MPR treatment:	18
1	29
3	196
6	306
24	324
48	461
96	290

NOTE: At the indicated times after injection of Me6MPR, groups of 3 mice were killed**, tumor cells from each group pooled, and PRPP content of the cells determined (132).

* 1.2 μmoles per mouse.

** On the 7th day after tumor implantation.

TABLE XXIV

Abolition by adenine of the Me6MPR-induced stimulation of 6MP anabolism in EAC cells *in vivo*

Pretreatment	Substrate	Total nucleotide-^{14}C (mμmoles per gram cells)
Saline	6MP-8-^{14}C*	109
Me6MPR	6MP-8-^{14}C	600
Me6MPR	6MP-8-^{14}C plus adenine	78
None	6MP-8-^{14}C plus adenine	12
None	Adenine-8-^{14}C	371
None	Adenine-8-^{14}C plus 6MP	425

NOTE: Tumor-bearing mice in groups of 3 were pretreated as indicated. Six hours later ^{14}C-labelled substrates, alone or together with nonisotopic substrates, were injected into the ascitic fluids; after 30 minutes, mice were killed and tumor cells from each group were pooled. Total labelled nucleotide formed in the tumor cells was then analyzed as described in MATERIALS AND METHODS.

* Dosages of drugs (μmoles per mouse) were as follows: 6MP (or 6MP-8-^{14}C), 3,3; Me6MPR, 1.2; adenine (or adenine-8-^{14}C), 5.8.

the test dose of 6MP.

It should be noted that while competition for PRPP appears to be the simplest explanation of this adenine effect, it is recognized that an alternative possibility exists. Although adenine nucleotides are unlikely to inhibit 6MP nucleotide synthesis, as has been mentioned, hypoxanthine and guanine nucleotides derived from adenine, if their concentrations were appreciably increased, could inhibit the hypoxanthine-guanine phosphoribosyltransferase (133,154), and thereby nullify the stimulatory effect of Me6MPR on 6MPRP synthesis. Thus, although the results of this experiment are consistent with the idea of increased availability of PRPP as a cause of the enhancement of 6MP nucleotide synthesis, these results do not actually prove this idea.

It is also seen in Table XXIV that without Me6MPR pretreatment, the synthesis of nucleotide from 6MP in the presence of adenine was 12 mμmoles per gram of cells in 30 minutes. Under identical conditions, the synthesis of nucleotide from adenine in the presence of 6MP was 425 mμmoles per gram cells in 30 minutes. Thus, the PRPP "available" (134) during the 30 minute period was about 437 (425 + 12) mμmoles per gram of cells. Since 600 mμmoles of 6MP nucleotide per gram cells was found in Me6MPR-pretreated cells in this experiment, it appears that the availability of PRPP is ordinarily a rate-limiting factor for 6MP nucleotide synthesis and that increased amounts are available to this reaction in Me6MPR-treated cells.

It is concluded from these experiments that the increase in the availability of PRPP is a likely basis for the enhancement of 6MP anabolism.

3. Metabolism of 6MP in Me6MPR-treated EAC cells

In most of these experiments, the synthesis of nucleotide from 6MP was measured 2 hours after injection of 6MP. After such an interval in EAC cells, the principal nucleotides derived from 6MP are 6-thioinosinate, 6-methylthioinosinate, and 6-thioxanthylate (50). To determine how the concentrations of these nucleotides varied with time, 6MP-8-^{14}C was injected into the ascitic fluids of mice bearing the Ehrlich ascites carcinoma, and after various time intervals, tumor cells were taken for analysis of the acid-soluble nucleotides by column chromatography on DEAE-Sephadex. It is seen in Figure 4 that the intracellular concentrations of the "total nucleotide" fraction and of 6MPRP reached a peak 30 minutes after injection of 6MP-8-^{14}C, and thereafter decreased with time. It is also seen in Figure 4 that 4.5 hours after injection of 6MP-8-^{14}C, the concentrations of total radioactive nucleotide and 6MPRP in Me6MPR-treated cells were still about twice the maximum values achieved in control cells. The concentrations of Me6MPRP and TXMP in Me6MPR-treated cells increased with time in accordance with suggestions that both compounds are metabolites of thioinosinate (49,51). The Me6MPRP content of control cells did not increase beyond 2 hours after injection of 6MP-8-^{14}C, and TXMP formation reached a maximum 1 to 2 hours after

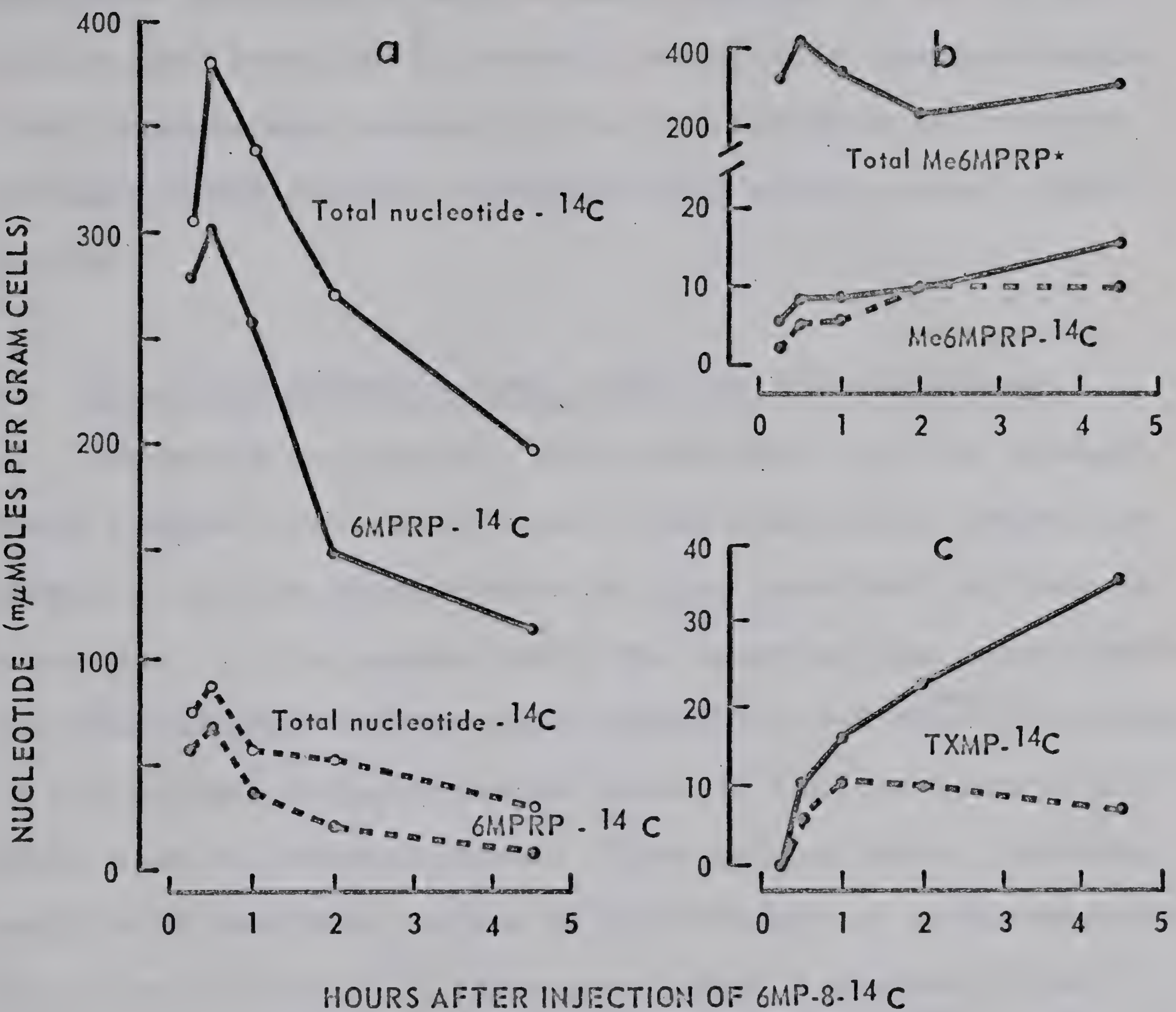
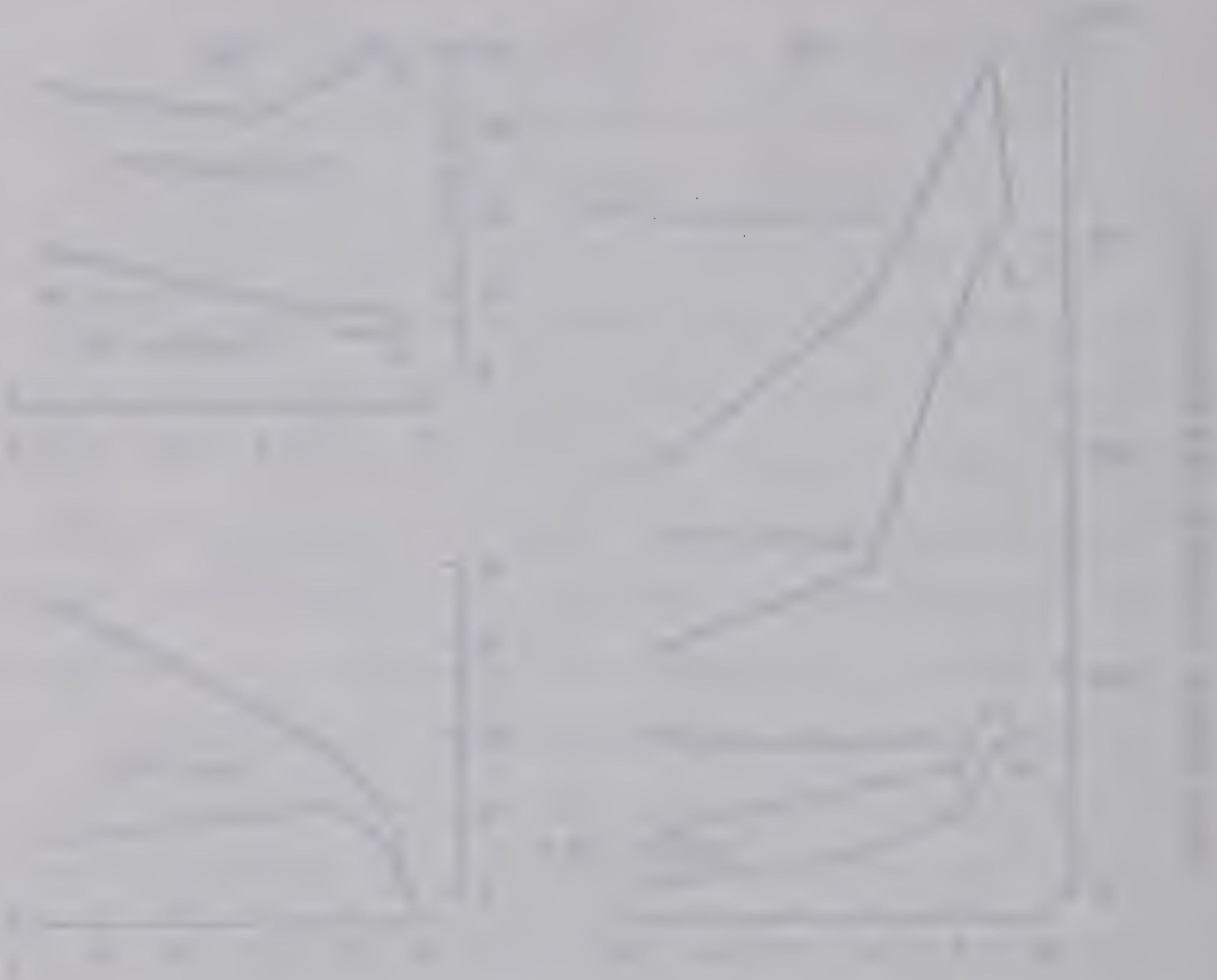


FIG. 4. Anabolism of 6MP in Me6MPR-pretreated EAC cells.

NOTE: Mice were used on the 7th day after implantation of EAC, 10 mice were each injected ip with 1.0 μmoles Me6MPR in 0.5 ml saline, and 10 other mice were each injected with 0.5 ml saline. After 6 hours, 1.4 μmoles of 6MP-8-^{14}C were injected into the ascitic fluid of each mouse; after the indicated time interval, 2 mice were withdrawn from each group and their tumor cells pooled. Perchloric acid extracts of each tumor cell sample were prepared and then analyzed by DEAE-Sephadex chromatography (121).

Solid lines indicate Me6MPR-pretreated cells and the broken lines indicate control cells.

* "Total Me6MPRP" was determined from OD_{292} data and, hence, represents Me6MPRP formed from both 6MP and Me6MPR.



injection of the isotope and then decreased.

It may be noted at this point that, although Me6MPR phosphate constituted only a small portion of the "total nucleotide" fraction in control cells, this compound would likely accumulate because of its low turnover and concentrations would increase substantially after several doses of 6MP.

C. Effect of Me6MPR on 6MP Anabolism in Mouse Liver

Henderson and Mercer (117) have shown that in several mouse tissues (including liver), the inhibitory effects of Me6MPR on purine biosynthesis *de novo* persisted as long as 24 hours. In the present work the question was asked whether the relationship between this inhibition and the stimulation of 6MP anabolism demonstrated above in ascites tumor cells might also be found in liver. Mice were injected intravenously with Me6MPR or saline 6 hours before an intravenous injection of 6MP-8-^{14}C and were killed 30 minutes later. Acid-soluble fractions of liver were then prepared and were analyzed by ion exchange chromatography on DEAE-Sephadex (121). In livers of both control and Me6MPR-treated animals the nucleotides formed from 6MP-8-^{14}C were 6MPRP, Me6MPRP, and an uncharacterized compound[14]. As can be seen in Table XXV, the concentrations of 6MPRP and the unknown metabolite in liver of Me6MPR-treated mice were about 5 times those of control mice, but concentrations of Me6MPRP formed

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[14] This compound appeared in the nucleotide region of the elution profile.

TABLE XXV

Effect of Me6MPR pretreatment on 6MP anabolism in mouse liver

	Nucleotides formed from 6MP-8-^{14}C (mμmoles per gram liver)			
Pretreatment	6MPRP	Me6MPRP	Unknown	Sum
NaCl	7	29	5	41
Me6MPR	37	32*	27	96

NOTE: Mice were injected intravenously with 1.2 μmoles Me6MPR or 0.5 ml 0.9% saline. After 6 hours each mouse was injected intravenously with 5.5 μmoles 6MP-8-^{14}C, killed 30 minutes later and the liver was quickly frozen in liquid nitrogen. The frozen livers from two mice of each group were placed together, weighed quickly, and powdered in a percussion mortar chilled with liquid nitrogen. The frozen powder was then extracted with cold perchloric acid. After neutralization, the extract was analyzed by DEAE-Sephadex chromatography (121).

* This value is based on ^{14}C data and, therefore, represents Me6MPRP derived from 6MP. Me6MPRP derived from the Me6MPR pretreatment was also present; based on OD measurement at 292 mμ, the total Me6MPRP concentration was 152 mμmoles per gram liver.

from 6MP were about the same in treated and control mice. The total amount of 6MP converted into nucleotide in the livers of Me6MPR-treated mice was twice that of control mice. Thus, these data show that 6MP anabolism in mouse liver was also stimulated by pretreatment with Me6MPR.

D. Effects of 6MP-Me6MPR Combinations on Glutamine-PRPP Amidotransferase

Since the inhibition of glutamine-PRPP amidotransferase has been implicated in the antineoplastic effects of 6MP and Me6MPR (37,63,102,103), the combined effects of these two agents on this enzyme was studied. In these experiments the activity of the amidotransferase was measured by the azaserine-induced accumulation of FGAR in intact tumor cells (135). The drug dosages used were within the therapeutic range. In Experiment A of Table XXVI, the dosages of Me6MPR employed alone and in combination with 6MP, caused almost complete inhibition of the enzyme. In Experiment B, it is seen that inhibition of the enzyme by the combination of 6MP and Me6MPR was less than that produced by Me6MPR alone. When a second drug treatment at the same dosage was given 24 hours later, the inhibition of the enzyme by two drugs together was increased by 7% over that caused by Me6MPR alone, as seen in Experiment C. These data indicate that when 6MP and Me6MPR were used together, the inhibition of glutamine-PRPP amidotransferase was mainly caused by Me6MPR; the contribution of 6MP to this inhibition, if any, was minor.

TABLE XXVI

Effect of 6MP and Me6MPR combinations on the glutamine-PRPP amidotransferase activity of EAC cells *in vivo*

Treatment	Dose (μmoles per mouse)	FGAR-^{14}C (mμmoles per gram cells)	Inhibition (%)
Experiment A (Single drug treatment)			
Control (saline)		48.3	0
6MP	3	18.1	63
Me6MPR	1.2	1.6	97
6MP plus Me6MPR	3 1.2	1.2	98
Experiment B (Single drug treatment)			
Control (saline)		45.3	0
6MP	1.2	38.8	14
Me6MPR	0.48	6.3	86
6MP plus Me6MPR	1.2 0.48	10.8	76
Experiment C (Two drug treatments, 24 hours apart)			
Control (saline)		47.0	0
6MP	1.2*	17.8	62
Me6MPR	0.48	6.2	86
6MP plus Me6MPR	1.2 0.48	3.4	93

NOTE: Tumor-bearing mice were injected ip, each with 0.5 mg azaserine**; purine analogues at the indicated dosages were injected ip into groups of 3 mice 30 minutes later. After a further 15 minutes, each mouse was injected with 2.5 μmoles glycine-^{14}C (uniformly labelled). One hour later, mice were killed, ascitic fluids from 3 mice in each group were pooled and tumor cells were extracted with perchloric acid. FGAR in the neutralized extracts was determined by the method of Henderson (135).

* Dose per treatment.

** In Experiment C, azaserine was injected in the second drug treatment.

E. <u>Enhancement of 6MP Anabolism as the Basis of the 6MP-Me6MPR Synergism</u>

If the Me6MPR-induced enhancement of nucleotide synthesis from 6MP increased the toxicity of 6MP toward EAC cells, then it would be expected that a simultaneous treatment of EAC cells with 6MP and Me6MPR would be less effective than treatment in which the same dosages are given separately with Me6MPR administered 6 hours before 6MP. This would be expected from results presented in a preceding section. In testing this idea experimentally, only single drug treatments could be used because Me6MPR phosphate, once formed, would persist in the tumor cells; thus, the effect on tumor mass of single drug treatments, given 24 hours after tumor implantation, were measured. Figure 5 shows that tumor cells exposed to the separate drug treatments proliferated at a significantly slower rate than those that received the simultaneous treatment. These data demonstrate a relationship between the Me6MPR-derived enhancement of nucleotide synthesis from 6MP (which also required <u>prior</u> Me6MPR treatment) and increased toxicity of 6MP toward EAC cells. Table XXVII presents the results of a similar experiment and shows that when an ineffective dose of 6MP was administered 6 hours after Me6MPR, potentiation of the drug effects resulted. However, when the same doses of 6MP and Me6MPR were administered together, no potentiation occurred. From these data it is concluded that the enhanced toxicity of 6MP toward tumor cells is apparently due to the Me6MPR-

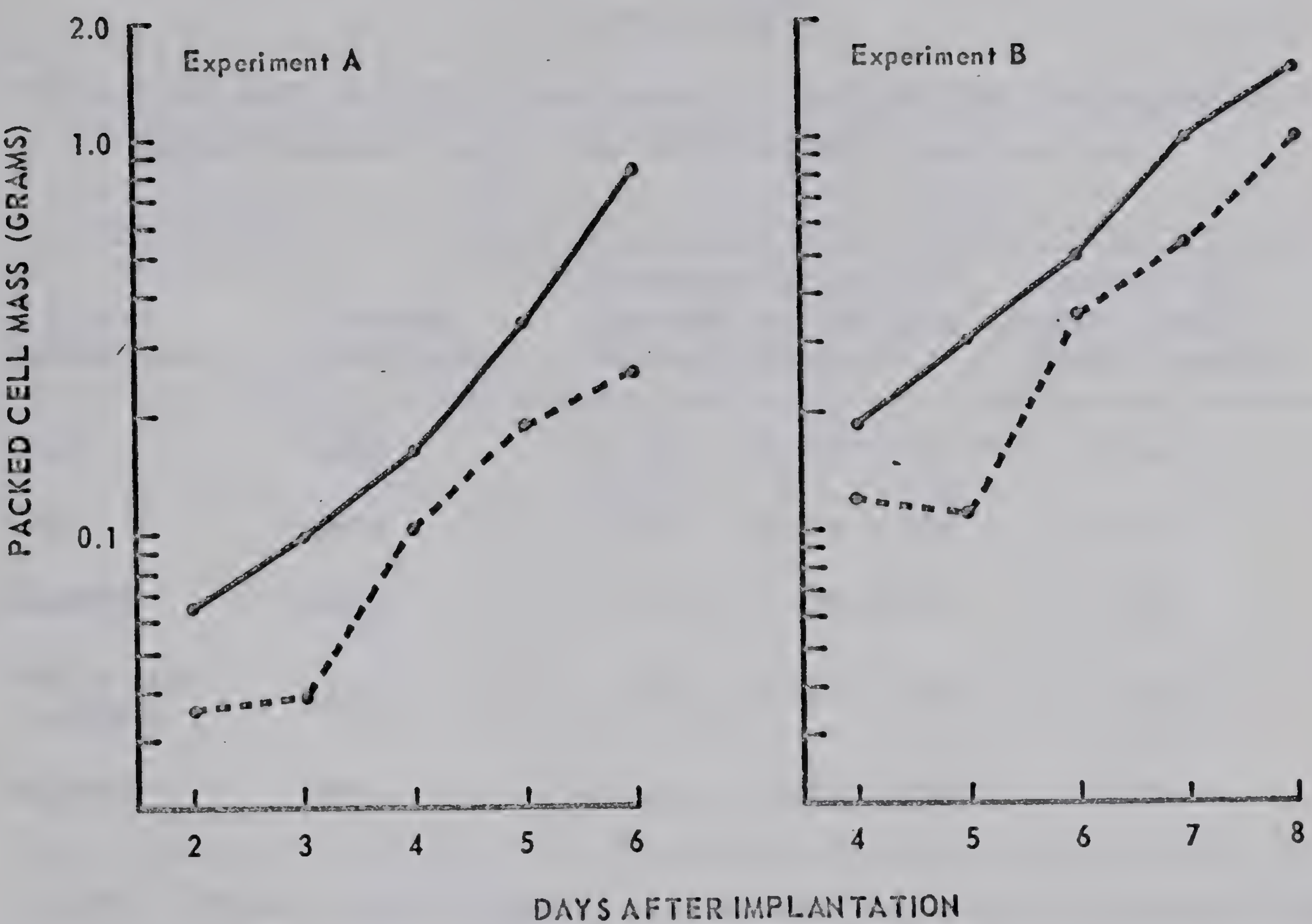

FIG. 5. Growth of EAC cells after simultaneous or separate treatments with 6MP and Me6MPR.

NOTE: 100 mice weighing 26-29 grams were implanted with EAC cells and randomly assigned to two equal groups. 24 hours after implantation, mice in the first group were injected ip with 1.2 μmoles Me6MPR plus 6 μmoles 6MP, and those in the second group were injected ip with 1.2 μmoles Me6MPR only. After 6 hours, mice in the first group received an ip injection of saline, and those in the second group received 6 μmoles 6MP.

After the growth periods indicated above, 10 mice were withdrawn from each group and the mass of the ascitic cells was determined; averaged values are plotted above.

Solid lines indicate simultaneous treatment and the broken lines separate treatment. Except for the first time point in Experiment A, the probability that the observed difference between two treatments occurred by chance was less than 5%, as calculated by Student's t-test.

TABLE XXVII

The importance of drug sequence in achieving therapeutic potentiation with the 6MP-Me6MPR combination

First treatment†	Second treatment††	Average mass of packed cells per mouse (grams)	Number of tumor cell doublings††
NaCl	NaCl	1.90 ± 0.55*(20)**	6.6
6MP	NaCl	1.83 ± 0.59 (10)	6.5
Me6MPR	NaCl	1.11 ± 0.33 (10)	5.8
6MP plus Me6MPR	NaCl	0.97 ± 0.33 (10)	5.6
Me6MPR	6MP	0.55 ± 0.17 (10)	4.5

NOTE: Mice (25-28 grams) received the first treatment 24 hours after tumor implantation and 6 hours later received the second treatment. The mass of tumor was measured 6 days after treatment.

The probability that the observed difference between simultaneous and separate treatments with 6MP and Me6MPR occurred by chance was less than 1%, as calculated by Student's t-test.

* Standard deviation.

** Number of mice.

† Dosages of drugs (μmoles per mouse per treatment) were as follows: Me6MPR, 1.2; 6MP, 6.

†† Calculated by method of Finney _et al_. (131).

induced stimulation of 6MP anabolism and that this is probably the basis for the 6MP-Me6MPR therapeutic potentiation.

Paterson and Moriwaki (8) have shown that 6MP and Me6MPR are therapeutically potentiating in the treatment of mouse lymphoma L5178Y. If the enhancement of 6MP anabolism by Me6MPR is truly a cause of the synergism, one could expect that 6MP anabolism would also be enhanced by Me6MPR in L5178Y cells. It is shown in Table XXVIII that the *in vivo* formation of nucleotides from 6MP in these lymphoma cells was indeed enhanced by Me6MPR.

TABLE XXVIII

Effect of Me6MPR on nucleotide synthesis from 6MP in L5178Y lymphoma cells *in vivo*

First treatment*	Second treatment*	Labelled nucleotides (mμmoles per gram cells)			
		6MPRP	Me6MPRP	TXMP	Total
6MP	6MP-8-^{14}C	43	9	28	82
6MP plus Me6MPR	6MP-8-^{14}C plus Me6MPR	114	0	47	186

NOTE: The first drug treatment was given on the 6th day after tumor implantation and the second was given 24 hours later. Mice were killed 2 hours after the second drug treatment and tumor cells from 5 mice in each group were pooled and extracted with perchloric acid. The extracts were then analyzed for individual nucleotides by DEAE-Sephadex chromatography (121), or for total labelled nucleotide by paper chromatography in solvent A, as described in MATERIALS AND METHODS.

* Dosage of 6MP (or 6MP-8-^{14}C) was 1.1 μmoles per mouse, that of Me6MPR was 0.4 μmoles per mouse.

IV. DISCUSSION

A. Combination Chemotherapy

Purine analogues have been used in conjunction with various kinds of drugs in the treatment of neoplastic disease, both experimental and human (1,136,137). Among these analogues, 6MP undoubtedly has drawn the most attention because it is effective against a wide spectrum of experimental tumors (12), and because it has the ability to induce remissions in human leukemia (1,14). In a number of instances, combination treatments involving 6MP have produced therapeutically potentiating effects. Concomitant use of 6MP and the glutamine antagonists, azaserine or 6-diazo-5-oxo-L-norleucine (DON), against experimental tumors are well-known examples of drug combinations that elicit synergistic growth inhibitory effect (5,138-142). As another important example of drug effects that cooperate, the xanthine oxidase inhibitor, HPP, has been shown to potentiate the inhibition of Adenocarcinoma 755 by 6MP when these drugs are used together (4). The concurrent use of 6MP and prednisone in the treatment of acute lymphatic leukemia of childhood has produced significantly higher rates of complete remission than would be expected from use of these drugs individually (1).

The present work has demonstrated that combination treatment of the Ehrlich ascites carcinoma with Me6MPR and

6MP resulted in a distinct potentiation of therapeutic effects. When these drugs were administered at low, non-toxic doses to mice bearing the Ehrlich ascites carcinoma, a high proportion of mice survived, apparently cured, whereas treatment with the individual drugs alone at the same dosages prolonged the survival time only slightly. Synergism with this drug pair was also demonstrated by direct measurement of tumor mass or volume. These results are very similar to other obtained with combinations of glutamine antagonists and 6MP in the treatment of Ehrlich ascites carcinoma (5), leukemias L1210 (141,142) and L5178Y (141,142) and Sarcoma 180 (139). AS will be discussed later, the biochemical bases of the therapeutic potentiation produced by 6MP-Me6MPR and by 6MP-azaserine (or DON) have some similarities.

It should be noted that in this work the potentiation resulting from the use of Me6MPR and 6MP in combination was demonstrated primarily by the production of long-term survivors, which is a qualitative criterion. The measurement or demonstration of synergism is further complicated by the contribution of the host in combating the tumor (143). It is likely that the host immune response may be involved in the final elimination of small numbers of tumor cells not killed by antitumor agents, because "cured" mice from such experiments have been found immune to reimplantation with the Ehrlich ascites carcinoma[15].

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[15] A.R.P. Paterson, personal communication.

Thus, use of the survival-time criterion in this particular system does not provide a rigid demonstration of results of the drug combination as "synergistic", according to the definition given above. However, when dosages of 6MP, which by themselves were without significant effect, were combined with Me6MPR (Table VI), the reduction in tumor volumes recovered from the treated animals was distinctly greater than that achieved by using Me6MPR alone. This indicates that the two drugs do, indeed, act in a synergistic manner against the Ehrlich ascites carcinoma. If 6MP and Me6MPR acted in an additive manner, one could expect that when an ineffective dosage of 6MP was used together with Me6MPR, the result would not be better than that produced by Me6MPR alone. Furthermore, Paterson and Moriwaki (8) have shown that cultured L5178Y lymphoma cells are synergistically inhibited by the Me6MPR-6MP combination, indicating that synergism can be elicited in the absence of host factors. In the latter studies, isobolic plots (as described by Hitchings (138)) were used to demonstrate that the cultured lymphoma cells were inhibited synergistically, not additively. In the same work it was also shown that L5178Y cells were inhibited synergistically _in vivo_ by the 6MP-Me6MPR combination.

Independently, Schabel _et al_. (7) have demonstrated that therapeutic potentiation takes place when Me6MPR and 6MP are used in combination in the treatment of mouse leukemia L1210. In their work, synergism was demonstrated with

a method which estimates the fractional kill of cells from the experimental parameter, extension of survival time. This method is based on the knowledge that the lethal cell burden (for L1210 cells in the BDF_1 mouse) is 10^9 cells, and assumes that drug treatment kills a constant proportion of cells instantly and that the surviving cells proliferate at the same rate as untreated cells; with this method, the proportion of cells killed by a drug dose is estimated by a graphical procedure. Schabel et al. showed that a combination of Me6MPR and 6MP, each at 50% of LD_{10}, killed a higher proportion of L1210 cells than did the individual drugs administered at the LD_{10} dosage. Their data also indicate that the fraction of cells killed by the above combination is greater than that expected from the sum of the individual drug effects at LD_{10} dosages.

Thus, the synergism resulting from the combination of 6MP and Me6MPR has been demonstrated in different tumor systems with different methods. At this point, one may ask whether combination treatment is really therapeutically beneficial to the tumor-bearing host. It is readily appreciated that the therapeutic value of a given combination treatment depends upon whether the result is better than the maximal effect of the individual drugs, within the limits of tolerable toxicity; in other words, if the potentiation of antitumor activity is accompanied by a substantial increase in host toxicity, the therapeutic advantage may be nullified. The latter view is well exemplified by the combination

treatment of L1210 leukemia with amethopterin and 6MP: even though preliminary studies by Skipper et al. (144) showed that the drugs in combination produced effects greater than those produced by either drug alone, Goldin et al. (145) found that this drug combination exerted a synergistic toxicity toward host animals. When treatment effects at fixed levels of host toxicity were compared, no synergism could be seen. Again, potentiation in the therapeutic sense is well exemplified in the work of Schabel et al. (7), who showed that at LD_{10} dosages, combination treatment with Me6MPR and 6MP prolonged the survival time of L1210-bearing mice to a greater extent than did individual drugs. The present work was mainly concerned with the biochemical basis of the potentiation of drug effects and no attempts have been made to optimize the dosages to prove that the combination has therapeutic gain.

It is apparent in the treatment of 4- and 7-day Ehrlich tumors (Tables VII and VIII) that the toxicity of the drug combination toward the tumor-bearing host limited in a very real way the therapeutic benefit that could be derived from treatment; however, this host toxicity did not entirely counterbalance the potentiative effect produced by the concomitant use of 6MP and Me6MPR. In these experiments, the combination of 6MP and Me6MPR at 32 and 20 mg/kg, respectively, killed 80% of the tumor-bearing mice, although these doses were only about 40% of the LD_{10} for tumor-free mice; these drugs individually at the same dosages killed not more

than 10% of tumor-bearing mice. At one half of the above dosages (i.e., at about 20% of LD_{10}), these drugs in combination produced (without lethal effects toward the host mice) a higher proportion of long-term survivors than did the individual drugs alone at doses which ranged approximately from 10-100% LD_{10} (for tumor-free mice) (Table VIII).

These data suggest combination therapy with 6MP and Me6MPR may have clinical usefulness; indeed Bodey *et al.* (9) have employed this treatment against acute myelogenous leukemia in adults and the results seem to be better than those obtained with 6MP alone, although more data are needed to prove this point.

B. Mechanism of the Synergism

1. Biochemical transformation of the drugs involved

It is known that the conversion of 6MP and Me6MPR to their respective nucleotides is an essential step before the manifestation of the antitumor activities of these drugs (37, 63,99,100). Intuitively, one would suppose that this conversion would also be essential for production of the synergism with the combination treatment. However, since both the 6MP and Me6MPR *per se* produce certain biochemical effects, as has been mentioned in the introduction, the possibility exists that these might be an essential part of the mechanism of the synergism. If this was true, then the synergism might be produced without "activation" of 6MP or Me6MPR. This possibility has been excluded by experiments in whcih

the combination of drugs failed to produce synergism against thiopurine-resistant tumors which have defects in converting either 6MP or Me6MPR to nucleotides. Hence, it is concluded that formation of drug nucleotides is an obligatory step in the manifestation of the synergism.

2. Enhancement of 6MP anabolism by Me6MPR

Since the formation of drug nucleotides was found to be an obligatory step in manifestation of the 6MP-Me6MPR synergism, this process was studied in EAC cells that had been exposed to both agents. It was found that phosphorylation of Me6MPR was not affected by 6MP, whereas the synthesis of nucleotide from 6MP was strongly stimulated by Me6MPR. The phosphorylation of the latter was evidently essential to the enhancement of 6MP anabolism because nucleotide synthesis from 6MP in EAC-R2 tumor cells was not significantly elevated by Me6MPR. These tumor cells are not able to phosphorylate Me6MPR. There are several possible mechanisms by which Me6MPR phosphate might stimulate nucleotide synthesis from 6MP:

a) Activation of hypoxanthine-guanine phosphoribosyltransferase: Hori *et al.* (146) reported that certain naturally-occurring nucleotides stimulated the activity of adenine phosphoribosyltransferase. This observation raised the possibility that Me6MPRP might enhance 6MP anabolism by acting as an allosteric effector of hypoxanthine-guanine phosphoribosyltransferase. However, time course studies indicated that after injection of Me6MPR, the maximal stimu-

latory effect of Me6MPR came later than the time at which maximum concentrations of Me6MPR phosphate were reached.

b) Reduction of catabolism of 6MP nucleotide: Thioinosinate is formed rapidly in Ehrlich ascites cells, but has a transitory existence only. The data of Paterson (54) suggest thioinosinate has a half life of about 1 hour in these cells. Thioinosinate may be dephosphorylated by the 5'-nucleotidase of these cells (147). It is possible that Me6MPR phosphate might act as a substrate analogue and thereby decrease the catabolism of thioinosinate. Such a possibility does not seem likely for the reasons stated above in (a). Furthermore, time course studies (Figure 4) show the rate of disappearance of 6MP nucleotide did not proceed at a lower rate in Me6MPR-treated cells than in untreated cells.

c) Increase in the amount of hypoxanthine-guanine phosphoribosyltransferase: If Me6MPR treatment derepressed the synthesis of this enzyme, then the rate of conversion of 6MP to nucleotide would be increased. However, it was found that in the extracts of Me6MPR-treated and untreated ascites tumor cells, the activities of the enzyme were similar. An analogous conclusion has been reached by Paterson (148) in his studies of the stimulatory effect produced by 6MP pretreatment on 6MP anabolism in Ehrlich ascites tumor cells *in vivo*.

d) Increased PRPP availability: Since the synthesis of thioinosinate requires PRPP, it is possible that PRPP

availability might ordinarily limit the rate of thioinosinate formation. The possibility was considered that an increased availability of PRPP would occur in the presence of Me6MPR phosphate because the latter is a potent inhibitor of glutamine-PRPP amidotransferase. There are several pieces of evidence to support the idea that 6MP anabolism was stimulated by this mechanism: (i) the PRPP level in Me6MPR-treated tumor cells was increased several-fold over that in control cells, (ii) the elevation of PRPP levels in tumor cells (which had been treated with one dose of Me6MPR) coincided in time with the intracellular occurrence of Me6MPR phosphate and with the enhancement of 6MP anabolism, (iii) other reactions that utilized PRPP, such as the synthesis of adenylate from adenine, were enhanced by pretreatment with Me6MPR, (iv) adenine abolished completely the stimulatory effect of Me6MPR, apparently by competing for PRPP, and (v) the availability of PRPP was found to be a rate-limiting factor for 6MP nucleotide synthesis in Ehrlich ascites tumor cells.

Although an increase in the availability of PRPP could reasonably result from blockage of the PRPP-consuming reaction of purine synthesis <u>de novo</u>, it is recognized that Me6MPR could possibly exert this effect by another mechanism. It has been reported that a number of nucleotides, particularly ADP, inhibited partly purified preparations of PRPP synthetase (155,156). Treatment of Ehrlich ascites tumor cells with Me6MPR leads to a decrease in intracellular concentrations of ADP, and ATP[16] and hence might release the synthetase

[16] J.M. Oliver, unpublished results.

from inhibitory actions of these compounds.

In addition, it should be noted that while increased availability of PRPP appears to be a possible basis for the enhancement of 6MP anabolism, another factor may also be involved in this respect. Guanine and hypoxanthine nucleotides are known to inhibit the activity of the hypoxanthine-guanine phosphoribosyltransferase (66,133,154), and if inhibition of purine synthesis *de novo* reduced intracellular concentrations of these compounds, as might be expected, an increase in the activity of the phosphoribosyltransferase might result with a consequent increase in the rate of 6MPRP synthesis.

3. Enhancement of 6MP anabolism as a cause of synergism

One of the possible mechanisms by which synergistic effects might result from the use of drugs in combination is through alterations in the metabolism and distribution of the drugs employed (136). It is readily appreciated that drug concentration in the target cell is a critical factor in the achievement of a drug effect. It is also known that certain drugs, for example, the purine analogues, must be converted to "active" forms to produce their effects; on the other hand, cellular detoxication mechanisms compete with activation processes by converting drugs to inactive forms. Thus, the effect of a given drug could be enhanced by a second agent in several ways. The entrance of a drug into cells or into an intracellular compartment might be accelerated by the presence of another drug, or the loss of drug from these might be prevented by changes in the intracellu-

lar environment (such as pH changes) caused by the second agent. Activation of a drug could be enhanced, or inactivation of a drug could be prevented, by a second agent.

The generally accepted idea that the synthesis of nucleotide from 6MP is an essential step for the antitumor activity (99,100) suggests that the enhancement of 6MP nucleotide formation by Me6MPR might possibly be a basis of the observed synergism. It was found in single dose treatments of the Ehrlich ascites carcinoma that both enhancement of the drug nucleotide formation and the potentiation of the drug effect required prior treatment of the cells with Me6MPR, suggesting that these two effects are related. In addition, enhancement of 6MP anabolism has also been seen in L5178Y cells, which are also synergistically inhibited by 6MP and Me6MPR. Mouse leukemia L1210 is another type of tumor cell which responds synergistically to chemotherapy with the 6MP-Me6MPR pair; it is not known whether 6MP anabolism in L1210 cells is enhanced by Me6MPR, although it has been shown that 6MP nucleotide synthesis in these cells is increased by prior treatment with 6MP[17]. It would appear that this 6MP-pretreatment effect occurs by a mechanism similar to that described above for the Me6MPR pretreatment effect, that is, Me6MPR phosphate derived from 6MP anabolism in the L1210 cell (35) may very well be responsible for the enhacement. The fact that single, simultaneous treatments of the L1210 leukemia _in vivo_ with 6MP and Me6MPR failed to produce a

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[17] M.L. Meloni and W.I. Rogers, personal communication.

potentiating effect (7), is in agreemtn with the observation obtained with EAC cells that on such treatment 6MP anabolism was not increased and the potentiation of drug effects was not produced.

From the foregoing considerations, it is concluded that the Me6MPR-induced enhancement of 6MP anabolism is likely to be a cause of the synergism produced by the combined use of these drugs in chemotherapy. The biochemical basis of this synergism may be similar in part to the synergism found with the 6MP (or 6-thioguanine): glutamine analogue combinations. The early steps of purine biosynthesis _de novo_ are strongly inhibited by the glutamine antagonists and in cells treated with these agents the conversion of 6MP (or 6-thioguanine) to nucleotide is enhanced (54,119,149). The therapeutic potentiation produced by the combination of 6-thioguanine and Me6MPR against mouse leukemia L1210 (150) may also have a similar biochemical basis to that presently described.

It should be noted that although the enhancement of 6MP anabolism is the probable cause of the synergism, such an increase would not necessarily ensure manifestation of the synergism. This is because mechanisms of 6MP resistance can operate not only to frustrate 6MP activation, but also at subsequent stages concerned with the action of 6MPRP. For example, 6MP-resistant lines of Adenocarcinoma 755 and _E. coli_ retain the ability to synthesize 6MPRP (27,56); in the latter instance, resistance was attributed to the ability of the bacterial cells to dethiolate 6MPRP to form IMP (27).

For these reasons, the fact that ETGRI subline was resistant to therapy with the 6MP-Me6MPR combination, even though nucleotide synthesis from 6MP was enhanced by Me6MPR, is not contradictory to the conclusion that Me6MPR-induced enhancement of 6MP anabolism is a probable cause of the synergism.

Since the enhancement of 6MP anabolism is associated with an increase in 6MP toxicity to the tumor cell, it might be asked whether a similar relationship existed at the host level and whether the Me6MPR effect on 6MP anabolism also occurs in normal cells. In this connection it was found that Me6MPR did, in fact, stimulate nucleotide synthesis from 6MP in mouse liver. Although no other tissues were examined in the present work, it is possible that in other normal tissues this same effect would occur; in this connection, Henderson and Mercer (117) have reported that Me6MPR inhibited purine synthesis _de novo_ in various mouse tissues _in vivo_. These considerations suggest that the combination of 6MP could potentiate host toxicity. The striking host toxicity produced by the 6MP-Me6MPR combination in the experiments of Tables VII and VIII might be due in part to a stimulation of 6MP anabolism in normal tissues. However, the enhancement of host toxicity does not necessarily mean that therapeutic gain will not be obtained by the combination of drugs.

This work is concerned only with the mechanism of the synergism, and does not indicate what biochemical effects produced by the drugs in combination are responsible for the lethal effects towards tumor cells.

Since the inhibition of glutamine-PRPP amidotransferase has been implicated in the antineoplastic effects of both 6MP and Me6MPR (37,63,102,102), one may imagine that the therapeutic potentiation might arise if this enzymatic activity was inhibited in a synergistic manner by these drugs. However, it was found that inhibition of the amidotransferase in Ehrlich ascites cells caused by therapeutic dosages of Me6MPR, was not increased significantly by additional treatment with 6MP. Atkinson[5] has found that thioinosinate, not only did not inhibit a partly purified preparation of the amidotransferase from the EAC cells, but actually antagonised the Me6MPR phosphate inhibitory effect.

Thus, inhibitory effects in addition to those at the amidotransferase site should be considered. In studies on the combination treatment of lymphoma L5178Y cells in culture, Paterson and Moriwaki (8) have found that Me6MPR reduced the proliferation rate of these cells, but did not kill them. In contrast, when 6MP was present in the culture medium the proliferation rate of the lymphoma cells progressively declined and the cells were killed in a time-dependent manner. 6MP alone, or in combination with Me6MPR showed a "delayed" toxic effect in these experiments; accordingly, it is possible that this lethal effect was caused by incorporation of 6MP, or its metabolites, into polynucleotides (see also section IB1d) and that this effect of 6MP was augmented by Me6MPR through the enhancement of 6MP anabolism. Other studies currently in progress in this laboratory have shown

that 6MP therapy also produces a delayed toxic effect on the proliferation of Ehrlich ascites tumor cells *in vivo*[7] and in culture[18].

The various inhibitory effects of thioinosinate in the area of nucleotide metabolism may also be important in the mechanism of the synergism, in view of the reported utilization by tumor cells of "preformed" purine bases derived from other tissues (151). The utilization of preformed bases constitutes an alternative to the "*de novo*" route, and therefore, affords a means of circumventing antimetabolite blockages on the latter route. Thioinosinate derived from 6MP would be expected to inhibit utilization of preformed purines through inhibition of purine nucleotide interconversions, and, again, this effect could be amplified by the Me6MPR stimulation of thioinosinate synthesis.

In conclusion, the enhancement of 6MP anabolism appears to be related to the observed therapeutic potentiation, but what biochemical effect produced by drugs in combination are responsible for the tumorcidal action is a matter that remains to be elucidated.

[18] L.J. Fontenelle, unpublished results.

V. SUMMARY

Thioinosine had only minor inhibitory effects on growth of the Ehrlich ascites carcinoma even when dosages were the molar equivalent of highly inhibitory 6MP dosages. However, when thioinosine was combined with Me6MPR, a distinct potentiation in therapeutic effect occurred. Evidently, 6MP was derived from 6MPR in achieving this synergism, but formation of the free base from Me6MPR was not involved, because Me6MP did not potentiate the antitumor activities of either 6MP or 6MPR.

Potentiation in growth inhibition resulting from the combination of 6MP with Me6MPR was demonstrated by comparing the effects of drug treatments on tumor cell mass (or volume) and on the survival times of tumor-bearing mice. The drugs in combination were synergistic, not only when chemotherapy was begun 24 hours after tumor implantation, but also when 4- and 7-day tumors were treated.

The combination treatment did not prolong the survival time of mice implanted with any of several thiopurine-resistant tumors; the latter had defects in the conversion of either 6MP or Me6MPR to their respective nucleotides, which are believed to be the "active" forms of these agents. From these findings it was concluded that nucleotide derivatives of both agents must be formed before the potentiation in therapeutic effect is manifested.

Experiments were undertaken to determine whether one member of the synergistic pair influenced the metabolism of the other. It was found that Ehrlich ascites tumor cells that had been pretreated in vivo with Me6MPR had an increased ability to synthesize thioinosinate from a test dose of 6MP. On the other hand, pretreatment with 6MP did not enhance the phosphorylation of Me6MPR in these cells. It was suspected that the enhancement of 6MP anabolism might be the basis for the therapeutic synergism. However, when the two drugs were injected simultaneously into the ascitic fluid of EAC-bearing mice to simulate combination chemotherapy, the synthesis of 6MPRP from the administered 6MP was not enhanced. This apparent contradiction was resolved when it was realized that a time interval was required for manifestation of the Me6MPR effect. This became apparent when it was shown that one treatment with the drug combination stimulated the anabolism in the tumor cells of the 6MP component of the next following drug treatment. The phosphorylation of Me6MPR was not enhanced in this situation. Subsequent experiments showed that this stimulatory effect of Me6MPR was maximal between 6 and 12 hours following pretreatment and was surprisingly long-lived, persisting up to 96 hours with little reduction.

The persistence of the stimulatory effect of Me6MPR correlated with the low turnover of Me6MPR 5'-phosphate in these cells. From these findings it appeared that the presence of Me6MPR 5'-phosphate in the tumor cells was responsi-

ble for the enhancement of 6MP anabolism. In agreement with these ideas it was found that in cells of the EAC-R2 subline (selected for resistance to Me6MPR and deficient in adenosine kinase) the synthesis of nucleotide from 6MP was not enhanced by Me6MPR pretreatment.

Me6MPR pretreatment did not enhance the anabolism of 6MP by increasing the concentration of the phosphoribosyltransferase that catalyses the PRPP-dependent synthesis of 6MPRP. Judged by the results of time course experiments, it appeared that this enzyme was not activated, or that 6MPRP breakdown was not inhibited, by the presence of intracellular pools of Me6MPR 5'-phosphate. It was found that, following injection of Me6MPR into the ascitic fluid of EAC-bearing mice, the maximum stimulatory effect on 6MP anabolism in the tumor cells came several hours after maximum concentrations of Me6MPR 5'-phosphate were achieved.

Several pieces of evidence suggest that Me6MPR phosphate stimulates 6MP nucleotide synthesis by blocking glutamine-PRPP amidotransferase, thereby increasing the availability of PRPP for 6MPRP synthesis: (a) the concentration of PRPP in the ascites tumor cells was elevated 10-25 times by Me6MPR pretreatment, (b) the elevation of PRPP levels in the tumor cells was coincident with the presence of Me6MPR phosphate in the tumor cells and with the Me6MPR-enhancement of 6MP anabolism; all three effects persisted for more than 95 hours, (c) other PRPP-requiring reactions were stimulated by Me6MPR pretreatment, (d) adenine, when administered together with

6MP, prevented the Me6MPR stimulatory effect on 6MP nucleotide synthesis, (e) availability of PRPP is ordinarily a rate-limiting factor for the synthesis of nucleotide from 6MP.

In agreement with the above ideas, Me6MPR administered by intravenous injection enhanced 6MP nucleotide synthesis in mouse liver; the glutamine-PRPP amidotransferase activity of this tissue is known to be inhibited by Me6MPR treatment *in vivo*.

A correlation was demonstrated between the two Me6MPR effects, the enhancement of 6MP anabolism and the potentiation of the antitumor activity. In inhibition of tumor cell proliferation *in vivo*, single simultaneous treatments with 6MP and Me6MPR were less effective than one treatment with Me6MPR, followed 6 hours later by a 6MP treatment. This result is consistent with the observation mentioned above, that Me6MPR treatment of ascites cells does not enhance 6MP anabolism unless it precedes the test doses of 6MP.

In other studies from this laboratory it has been shown that 6MP and Me6MPR synergize in inhibiting the proliferation of L5178Y lymphoma cells *in vivo* and in culture. In the present studies, it was found that nucleotide synthesis from 6MP in L5178Y cells was also enhanced by Me6MPR pretreatment.

When 6MP and Me6MPR were injected together into the ascitic fluids of tumor-bearing mice, the inhibition of glutamine-PRPP amidotransferase was not significantly greater than that produced by Me6MPR alone. Thus, inhibition at loci other than the amidotransferase are probably involved in the mechanism of the synergism.

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